To investigate the effect of the major UV-induced lesions on SV40 origin-dependent DNA replication and mutagenesis in a mammalian cell extract, double-stranded plasmids containing a single cis,syn-cyclobutane dimer or a pyrimidine-pyrimidone (6-4) photoproduct at a unique TT sequence have been constructed. These plasmids have been used as templates in DNA replication-competent extracts from human HeLa cells. Plasmids containing a single pyrimidine cyclobutane dimer on the potential lagging strand for DNA replication are replicated with an efficiency approximately equal to that of an unmodified plasmid. A small decrease in replication efficiency of ~20% was observed when the lesion was located on the potential leading strand for DNA replication. In both orientations, DpnI-resistant, replicated closed circular plasmid DNA was sensitive to nicking by the pyrimidine dimer-specific enzyme, T4 endonuclease V, indicating that complete replication of the damaged plasmid occurs in vitro. In contrast, a (6-4) photoproduct, within the same site and sequence context on the lagging strand for DNA synthesis, inhibits replication in vitro by an average of ~50% indicating that the mammalian replication complex responds differently to the two major UV-induced lesions during DNA replication in vitro. Analysis of the DpnI-resistant, replicated DNA for mutations targeted to the lesion site indicates that neither of these lesions resulted in significant mutagenesis. UV-induced lesions at TT sites may therefore be poorly mutagenic under these conditions for DNA replication in human cell extracts in vitro.

Exposure to the ultraviolet component of sunlight is a significant risk factor in the development of skin cancers, especially squamous cell carcinomas and, to a lesser extent, melanomas (1). While the mechanisms underlying the development of these cancers are complex, it has been observed that skin cancer cells from both humans (2) and rodents (3) show base changes in the sequences of critical genes, such as the p53 tumor suppressor gene, that have the characteristics of UV-induced mutations. In particular, the mutations in these genes are primarily at dipyrimidine sequences, potential sites of UV-induced DNA damage. The occurrence of tandem double-base CC → TT transitions is also more common in mutated p53 genes from skin tumors than in mutated p53 from tumors of internal organs (4). These results indicate that skin carcinogenesis may involve the conversion of UV-induced genomic DNA damage into base changes during the process of replication of damaged DNA. However, the molecular mechanisms by which damaged DNA is replicated and mutations are fixed are not well understood. The major types of DNA damage induced by UV irradiation occur at dipyrimidine sequences; these are cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts. A smaller proportion of thymine and cytosine hydrates is also formed (5). Thus, random UV irradiation of plasmid DNA or of cells in culture leads to the formation of a variety of photoproducts involving thymines, thymine and cytosine, and cytosines. The relative contribution of the various lesions to the inhibition of DNA synthesis and to mutagenesis has therefore been difficult to determine. In addition, studies using cultured cells may be complicated by the effects of DNA repair, which can vary in a site- and DNA strand-specific manner (6, 7).

UV irradiation of mammalian cells leads to a transient inhibition of DNA synthesis (8–10), which may allow time for the repair of potentially mutagenic lesions in the template before replication proceeds (11). While it has been proposed that this inhibition of DNA synthesis is due to the blockage of replication forks at the sites of DNA damage (12, 13), there is also evidence for inducible responses that are involved in the inhibition of initiation of new origins of replication (14, 15). In addition, DNA polymerase activity may also be inhibited by direct means following irradiation of cells, for example by the induction of the p21 protein, which results in the inhibition of processive DNA synthesis by DNA polymerase δ (16).

To directly determine the effect of a single UV-induced lesion in DNA on the mammalian replication complex, we have constructed plasmid molecules that contain either a single cis,syn-cyclobutane pyrimidine dimer or a (6-4) photoproduct at a unique TT site and used these plasmids as templates for DNA replication in vitro in extracts from human HeLa cells. It has been shown previously that mutagenic replication of randomly UV-damaged DNA occurs in these extracts (17, 18). By utilizing specifically modified plasmid derivatives, we demonstrate that complete replication past a UV-induced lesion occurs in vitro. Previous studies using site-specifically modified constructs in bacterial (19–21) and yeast (22, 23) systems have shown that these UV-induced lesions can differ substantially in their mutagenic potential. The cell-free approach described here allows the effect of specific lesions on DNA replication and mutagenesis to be studied under conditions in which the complete replication complex is active rather than individual DNA polymerases and repair of these lesions is essentially absent.

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† Thetrivial name and abbreviation used are: pyrimidine-pyrimidone (6-4), 5-hydroxy-6-4-pyrimidin-2-one-thymine; HPLC, high pressure liquid chromatography.
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(17) Using this approach, we show that the mammalian cell replication complex responds differently to a pyrimidine dimer and a (6-4) photoproduct at the same site and sequence context, with the (6-4) photoproduct being substantially more inhibitory to DNA replication in vitro that the pyrimidine dimer. However, neither lesion results in significant mutagenesis at the TT site following DNA replication in vitro.

EXPERIMENTAL PROCEDURES

Materials

T4 DNA ligase, T4 polynucleotide kinase, Taq DNA polymerase, EcoRI, and BglII were obtained from Life Technologies, Inc. AatII and BsaHII were from New England Biolabs Inc. (Beverly, MA). DpnI was from Boehringer Mannheim. T4 endonuclease V was obtained from Applichem. MboII and DpnI were obtained from New England Biolabs Inc. (Milwaukee, WI). Primers used in DNA sequencing and primer extension were synthesized on an Applied Biosystems oligonucleotide synthesizer. [α-32P]dCTP and [γ-32P]ATP were from DuPont NEN. Pipernine was obtained from Eastman Kodak Co.

Methods

Cell Culture and Extract Preparation—Mammalian HeLa cells were grown in Dulbecco's minimal essential medium at 37 °C and 5% CO2. Cell-free extracts for DNA replication in vitro were prepared by the method of Li and Kelly (24) as described previously (17).

Preparation of Specifically Modified Oligonucleotides—Oligonucleotides containing a single cis-syn-TT cyclobutane pyrimidine dimer were prepared by the method of Banerjee et al. (19, 20). The pyrimidine cyclobutane dimer was introduced into the 11-mer oligonucleotide 5'-GCAAGTTGGAG-3' by irradiation with 18 MJ/m2 320 nm UV radiation from a West Hunghouse FS40 sun lamp in an anoxic aqueous solution containing 20 μM acetophenone (19, 20). The oligonucleotide that contained a cis-syn-cyclobutane dimer was digested with the single-cuts enzyme T4 endonuclease V, formation of a 388-base fragment following piperidine treatment. The DNA was analyzed by electrophoresis on a 1% agarose minigel alongside known amounts (5–25 ng) of pZ189 plasmid that had been linearized by treatment with EcoRI. After ethidium bromide staining, the relative fluorescence intensity of the standard bands was quantitated using GPTools Version 3.0 (BioPhotonics, Ann Arbor, MI). These values were used to construct a standard curve of fluorescence intensity versus DNA amount. The amount of DNA in each lane was determined by transmission fluorescence intensity of the construct bands and this standard curve.

Analysis of Plasmid Constructs—The isolated plasmid DNA was analyzed for the presence of a cyclobutane pyrimidine dimer by treatment with the enzyme T4 endonuclease V. DNA was incubated with 1 unit of enzyme in 50 mM H2PO4, pH 6.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 μg of bovine serum albumin for 2 h at 37 °C. The DNA was analyzed by electrophoresis on a 1% agarose gel containing 5 μg/ml ethidium bromide. Conversion of closed circular (Form I V) DNA to nicked circular (Form II) DNA was detected by a change in mobility by UV transillumination and photography.

The exact position of the lesion in the plasmid construct was also mapped by primer extension analysis. UV-induced lesions in the DNA template were detected by DNA synthesis with a [32P]dNTP of the polymerase chain reaction (29). Construct DNA was incubated with 2.5 units of Taq polymerase in 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.01% gelatin, and 200 μM dNTP, with 0.3 pmol of the 5'-32P-labeled primer 5'-TATGTTTTTTACTGG-3', which anneals 81 bases 3' to the potential lesion site. Linear amplification was carried out in the polymerase chain reaction for 40 cycles of 30 s each at 95, 45, and 72 °C. The products were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel, followed by autoradiography of the dried gel. A second method was also used to confirm the position of the pyrimidine cyclobutane dimer in the plasmid construct. Plasmid DNA was digested with the single-cut enzyme BsaHI, which cuts at a position downstream from the lesion. The linearized DNA was labeled at the 5'-end with [α-32P]ATP using T4 polynucleotide kinase. Following treatment with T4 endonuclease V, formation of a 388-base fragment was analyzed by 6% denaturing polyacrylamide gel electrophoresis and detected by autoradiography of the dried gel. To detect the presence of the (6-4) photoproduct in the plasmid construct, primed by the 6-4 lesion, the DNA was treated by BsaHI and then labeled at the 5'-end with [γ-32P]ATP. The 5'-labeled DNA was either left untreated or treated with T4 endonuclease V as described or with 1 μm piperidine for 30 min at 90 °C. The DNA was recovered by ethanol precipitation, and any remaining piperidine was removed by drying overnight in a Savant Speedvac vacuum evaporator. DNA fragments were detected by denaturing polyacrylamide gel electrophoresis, followed by autoradiography of the dried gel. The presence of a (6-4) lesion in the DNA resulted in the formation of a 32P-labeled 388-base fragment following piperidine treatment.
Replication of the Modified Plasmid DNA in Vitro—DNA replication reactions were carried out (in 30 μl) using 60 μg of HeLa cell extract protein (10, 17); 25 ng of DNA template; 30 mM HEPES (pH 7.5); 7 mM MgCl₂; 0.5 mM dithiothreitol; 100 μM each dATP, dGTP, and dTTP; 50 μM dCTP; 10 μCi of [α-32P]dCTP; 200 μM each GTP, UTP, and CTP; 4 mM ATP; 40 mM creatine phosphate; 10 μg of creatine kinase; and 1 μg of SV40 large T antigen. Reactions were incubated for 3 h at 37°C, and the reaction products were isolated and purified as described previously (17, 30). Replication products were treated with the restriction enzyme DpnI to remove unreplicated DNA (17, 30), and the reaction products were separated by electrophoresis on a 1% agarose gel containing 5 μg/ml ethidium bromide. Replicated DNA was visualized by autoradiography of the dried gel. In the case of the pyrimidine cyclobutane dimer-containing DNA, this lesion was detected in the DpnI-treated DNA by treatment with 1 unit of T4 polynucleotide kinase, and the reaction product was treated with the restriction enzyme EcoRI and washed membranes. Plasmid DNA was isolated from colonies that failed to hybridize at the higher temperature and was analyzed by DNA sequencing of these plasmids prior to use in hybridization analysis. Colonies were transferred to filters and washed as described above. Hybridization in all experiments was detected by autoradiography of the washed membranes. Plasmid DNA was isolated from colonies that failed to hybridize at the higher temperature and was analyzed by DNA sequencing by the dideoxy chain termination procedure (31) with the primer (5'-TAATGTCTTTTACTGG-3') described above.

RESULTS

Construction of Specifically Modified Plasmids—Plasmids containing either a single cyclobutane dimer or (6-4) photoproduct were constructed as outlined in Fig. 1. Vector DNA was prepared from pZ189 or pZ189R2 by cleavage with EcoRI, dephosphorylation of the 5'-ends, and subsequent cleavage with AatII. Purification of the resulting 5430-base pair fragment yields a vector with a non-ligatable EcoRI site and a ligatable AatII site. The 11-mer oligonucleotide containing the appropriate UV-induced lesion at the TT site was phosphorylated at the 5'-end and annealed to an unphosphorylated complementary 19-mer, yielding a double-stranded linker with a ligatable AatII site and a non-ligatable EcoRI site. Insertion of this linker between the EcoRI and AatII sites of the vector was carried out in two steps. In the first step, the linker was annealed and ligated to the vector DNA at 20 ng/μl. Under these conditions, ligation occurs only at the AatII site, forming
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**Fig. 2. Analysis of the pyrimidine dimer-containing plasmid DNA.** A, constructs, prepared as outlined in Fig. 1 and purified by electrophoresis, were analyzed by agarose gel electrophoresis in the presence of 5 μg/ml ethidium bromide. Form IV, completely closed circular plasmid DNA; M, pZ189 marker plasmid; N, plasmid constructed using an unmodified linker; CPD, plasmid constructed using a TT pyrimidine dimer-containing linker. B, unmodified and pyrimidine dimer-containing constructs were treated with T4 endonuclease V (T4 endo V) at 37°C for 2 h. The DNA was purified by extraction with phenol, followed by ethanol precipitation. DNA was analyzed by electrophoresis on a 3% agarose gel in the presence of 0.2 μg/ml ethidium bromide and isolated by electroelution. The majority of the isolated DNA was in the completely closed circular form, as shown by analysis of an aliquot of the electroeluted DNA on a second 1% agarose gel containing 5 μg/ml ethidium bromide (Fig. 2A). To confirm that the purified closed circular plasmid DNA isolated from the agarose gel contained a pyrimidine dimer, an aliquot was treated with T4 endonuclease V, which introduces specific nicks to DNA at the site of this lesion. Nicking alters the mobility of Form IV DNA to that of nicked circular Form II DNA. The closed circular plasmid construct with the unmodified linker was insensitive to nicking by this enzyme, while the plasmid containing the cyclobutane dimer was sensitive to nicking (Fig. 2B). While some nonspecific nicks were introduced during processing into both the unmodified and modified plasmids, as evidenced by an increase in the proportion of Form II DNA (Fig. 2B), this does not account for the sensitivity of the modified construct to T4 endonuclease V (Fig. 2B). A small amount of linear vector DNA was observed after treatment of the dimer-containing plasmid with T4 endonuclease V; this could arise from molecules with more than a single insert. Nicking by T4 endonuclease V was inhibited by pretreatment of the modified DNA with E. coli DNA photolyase (a gift of Dr. A. Sancar, University of North Carolina), indicating that the endonuclease specifically recognizes a cyclobutane dimer under these conditions (data not shown). The presence of the adduct was confirmed by primer extension analysis. DNA synthesis by Taq DNA polymerase was blocked by the presence of this lesion (Fig. 2C, second lane), while a full-length product was observed with the unmodified construct (first lane). The blockage of synthesis occurred at the expected TT dipyrimidine position within the 19-mer insert in the plasmid sequence, by alignment with a sequencing ladder of this region of the plasmid run in parallel on the same gel (Fig. 2C, right panel). These data demonstrate that the constructs contain the appropriate inserts, corresponding to the schematic shown in Fig. 1.

**Analysis of the (6-4) Photoprodut-containing Plasmid—** To analyze closed circular DNA prepared from ligation reactions using the (6-4) photoprodut-containing linker (Fig. 3A) for the presence of this lesion, the DNA was first cleaved at a site 388 base pairs from the lesion by treatment with the single-cut enzyme BsaHI and labeled at the 5’-end using [γ-32P]ATP. The DNA was then treated either with 1 M piperidine at 90°C for 30 min, which cleaves DNA at (6-4) photoproducts, or with T4 endonuclease V for 2 h at 37°C to test for cyclobutane pyrimidine dimers, followed by denaturing polyacrylamide gel electrophoresis and autoradiography. This analysis indicated that a linear molecule with an EcoRI site at each end. The EcoRI sites were phosphorylated, and another ligation was carried out at a DNA concentration of 0.2 ng/μl, which favors intramolecular ligation or recircularization. In a low percentage of molecules, three linkers were inserted into the vector DNA, probably as a result of self-ligation of the linkers during the first ligation step, prior to recircularization (see below). Following the dilute ligation, the relaxed closed circular (Form IV) DNA products were separated from nicked circular (Form II) and linear products by electrophoresis on an agarose gel run in the presence of 5 μg/ml ethidium bromide and isolated by electrophoresis.

Analysis of the TT Pyrimidine Dimer-containing Plasmid—Relaxed, completely closed circular (Form IV) plasmids containing either an unmodified insert or a single pyrimidine dimer at the TT site (constructed as outlined in Fig. 1) were isolated from the gel by electroelution. The majority of the isolated DNA was in the completely closed circular form, as shown by analysis of an aliquot of the electroeluted DNA on a second 1% agarose gel containing 5 μg/ml ethidium bromide (Fig. 2A). To confirm that the purified closed circular plasmid DNA isolated from the agarose gel contained a pyrimidine dimer, an aliquot was treated with T4 endonuclease V, which introduces specific nicks to DNA at the site of this lesion. Nicking alters the mobility of Form IV DNA to that of nicked circular Form II DNA. The closed circular plasmid construct with the unmodified linker was insensitive to nicking by this enzyme, while the plasmid containing the cyclobutane dimer was sensitive to nicking (Fig. 2B). While some nonspecific nicks were introduced during processing into both the unmodified and modified plasmids, as evidenced by an increase in the proportion of Form II DNA (Fig. 2B), this does not account for the sensitivity of the modified construct to T4 endonuclease V (Fig. 2B). A small amount of linear vector DNA was observed after treatment of the dimer-containing plasmid with T4 endonuclease V; this could arise from molecules with more than a single insert. Nicking by T4 endonuclease V was inhibited by pretreatment of the modified DNA with E. coli DNA photolyase (a gift of Dr. A. Sancar, University of North Carolina), indicating that the endonuclease specifically recognizes a cyclobutane dimer under these conditions (data not shown). The presence of the adduct was confirmed by primer extension analysis. DNA synthesis by Taq DNA polymerase was blocked by the presence of this lesion (Fig. 2C, second lane), while a full-length product was observed with the unmodified construct (first lane). The blockage of synthesis occurred at the expected TT dipyrimidine position within the 19-mer insert in the plasmid sequence, by alignment with a sequencing ladder of this region of the plasmid run in parallel on the same gel (Fig. 2C, right panel). These data demonstrate that the constructs contain the appropriate inserts, corresponding to the schematic shown in Fig. 1.

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the construct prepared from the unmodified linker was insensitiver to both treatments since no labeled fragment was observed in the region of 300–400 bases on the gel (Fig. 3B, lane 10). Unmodified (lanes 1, 3, and 5) and (6-4) lesion-containing (lanes 2, 4, and 6) DNA samples were then processed in parallel reactions and either left untreated (lanes 1 and 2) or treated with 1 M piperidine (lanes 3 and 4) or with T4 endonuclease V (T4 endo V; lanes 5 and 6). As a control for T4 endonuclease V activity, unmodified (lanes 7 and 9) and pyrimidine dimer-containing (lanes 8 and 10) construct DNAs were also treated with enzyme buffer (lanes 7 and 8) or with T4 endonuclease V (lanes 9 and 10). DNA was then separated by electrophoresis on a 6% polyacrylamide gel, followed by autoradiography of the dried gel. M, 5′-32P-labeled 123-base pair marker. Numbers on the left refer to the sizes of the marker fragments in bases. The arrow indicates the position of the 388-base fragment generated by cleavage of the DNA at the site of the (6-4) photoproduct or the cyclobutane pyrimidine dimer.

In contrast, the construct prepared using the modified linker was sensitive to piperidine treatment as indicated by the presence of a band of 388 bases in the autoradiogram (Fig. 3B, lane 10), but not with the corresponding plasmid derived using the unmodified linker (lane 9).

Effect of a Single Cyclobutane Pyrimidine Dimer on DNA Replication in Vitro—Plasmid constructs, either unmodified or containing a single pyrimidine cyclobutane dimer on the potential lagging strand for DNA replication (pZ189-TT construct), were incubated in parallel reactions under the conditions for in vitro DNA replication in the presence and absence of SV40 large T antigen. DNA synthesis was monitored by measurement of the incorporation of radioactivity into trichloroacetic acid-precipitable material (17, 30). The kinetics of incorporation were very similar between the unmodified and singly modified templates (Fig. 4). The level of incorporation (4–5 pmol of dCMP) is in the range of the values previously reported for DNA replication in reactions containing 25 ng of template DNA (10, 17). Our previous analysis of the replication of un-

The construct prepared from the unmodified linker was insensitive to both treatments since no labeled fragment was observed in the region of 300–400 bases on the gel (Fig. 3B, lanes 3 and 5). T4 endonuclease V treatment of the construct prepared from the modified linker resulted in a very weak cleavage reaction (Fig. 3B, lane 6), indicating that this construct contains at most only a very low percentage of molecules that carry a pyrimidine dimer rather than a (6-4) adduct at the modification site. As a control for the activity of T4 endonucle-

The DNA was digested with nuclease P1 and either left untreated (lanes 2, 4, and 6) or treated with 1 M piperidine (lanes 3 and 4). The digested DNA was then separated by electrophoresis on a 6% polyacrylamide gel, followed by autoradiography of the dried gel. M, 5′-32P-labeled 123-base pair marker. Numbers in the left refer to the sizes of the marker fragments in bases. The arrow indicates the position of the 388-base fragment generated by cleavage of the DNA at the site of the (6-4) photoproduct or the cyclobutane pyrimidine dimer.
damaged and randomly UV-damaged pZ189 plasmids under these conditions in vitro, using density gradient centrifugation of plasmid DNA replicated in the presence of bromodeoxyuridine triphosphate, indicated that this level of incorporation is consistent with a single round of replication occurring in vitro (17). In those experiments, the majority of the radioactively labeled plasmid was found to sediment with a density corresponding to that of heavy-light DNA, consistent with a single round of replication occurring in vitro on both the undamaged and UV-irradiated plasmids (17). This previous observation, coupled with the facts that the majority of the incorporation of radioactivity into plasmid DNA in the present experiments is dependent on the presence of SV40 large T antigen in the reaction, and that the DNA labeled in a large T antigen-dependent manner is largely resistant to digestion by DpnI, indicates that the level of incorporation described here represents bona fide DNA replication. In addition, the results obtained using T4 endonuclease V treatment to analyze the replicated DNA (see below) strongly argue that both strands of the plasmid are replicated under these conditions.

In the absence of SV40 large T antigen in the reaction, the incorporation of radioactivity was low, indicating that DNA repair-type synthesis is not occurring to a significant extent, consistent with previous observations on UV-irradiated DNA incubated in this type of cell extract (Ref. 17 and Fig. 4). To examine the replication reaction in more detail, the DNA products were purified and analyzed by agarose gel electrophoresis in the presence of 5 μg/ml ethidium bromide, followed by autoradiography of the dried gel. The results indicate that in the absence of large T antigen, a low background of radioactivity is incorporated into both the unmodified and dimer-containing Form I DNAs (Fig. 5A, first and third lanes). The major band of the incorporation of radioactivity into the plasmid DNA was dependent on the presence of large T antigen in the reaction (Fig. 5A, second and fourth lanes), indicating that this represents DNA replication. To test whether this was the case, the products of DNA replication reactions carried out in the absence or presence of large T antigen were treated with the enzyme DpnI. Unreplicated input plasmid DNA, which carries the bacterial methylation pattern at 5′-GATC-3′ sequences, is sensitive to digestion by this enzyme. In contrast, any DNA that becomes completely replicated in vitro is resistant to digestion since the mammalian cell extract lacks the enzymes that become completely replicated sensitive to digestion by this enzyme. In contrast, any DNA the bacterial methylation pattern at 5′-GATC-3′ sequences, is resistant or presence of large T antigen were treated with the enzyme DpnI, consistent with the conclusion that this synthesis represents replication rather than repair-type synthesis (Fig. 5B).

The low level of labeled DNA observed with both constructs in the absence of large T antigen was sensitive to digestion by DpnI, indicating it represents background incorporation and not complete plasmid replication (Fig. 5B, first and third lanes).

Sensitivity of Replicated DNA to T4 Endonuclease V—If the plasmid DNA containing a single pyrimidine dimer becomes completely replicated in vitro, the labeled closed circular products, detectable following agarose gel electrophoresis, should be sensitive to nicking by the enzyme T4 endonuclease V. To investigate whether this was the case, the DpnI-resistant products of replication in vitro were treated with this enzyme. Sensitivity to nicking was determined by a change in the mobility of the labeled, completely closed circular (Form I) DNA to that of nicked circular (Form II) DNA following electrophoresis on a 1% agarose gel containing 5 μg/ml ethidium bromide (Fig. 6, A, second and fourth lanes; and B, second and fourth lanes). Fig. 6A shows results obtained with the construct (pZ189-TT) in which the dimer was on the potential lagging strand for DNA replication. Densitometric analysis of the data shown in Fig. 6A (compare third and fourth lanes) revealed that ~45% of the Form I molecules were sensitive to nicking. This level of sensitivity to T4 endonuclease V is consistent with a single round of replication occurring in vitro (17), resulting in the formation of two populations of daughter molecules. One population consists of molecules in which the parental strand is the dimer-containing strand, and is sensitive to nicking by T4 endonuclease V; in the other population, the parental strand is damaged-free and is therefore insensitive to nicking by T4 endonuclease V. The sensitivity of a large fraction of the DpnI-resistant, replicated Form I DNA to nicking by T4 endonuclease V argues that templates containing the pyrimidine dimer lesion can be replicated under these conditions.

To examine whether replication in vitro was influenced by the position of the adduct on the leading or lagging strand for DNA replication, a construct in which the pyrimidine dimer adduct was placed in the opposite orientation relative to the SV40 origin of replication (in pZ189R2-TT), such that it would be on the potential leading strand for DNA replication, was also used as a template. When using this template, the extent of replication, as determined from the level of incorporation of radioactivity from [α-32P]dCTP into trichloroacetic acid-precipitable material, was ~80% of that of the unmodified construct. While this is a small difference, these results suggest that a
single pyrimidine dimer may have a slightly greater inhibitory effect on DNA replication when located on the leading strand for DNA replication compared to when it is located on the lagging strand. However, a more detailed analysis of the replication of plasmids in which the dimer is located at different positions relative to the SV40 origin of replication will be required to fully elucidate the effect of this lesion on leading versus lagging strand DNA replication.

The identity of the DpnI-resistant, replicated DNA was confirmed by digestion of the replicated DNA with the enzyme BglI, which generates fragments of 4396 and 1048 base pairs in the normal orientation (in which the lesion is on the potential lagging strand) (Fig. 6C, first and second lanes), and of 3406 and 2038 base pairs in the reverse orientation (in which the lesion is on the potential leading strand) (Fig. 6C, third and fourth lanes).

To determine if the plasmid containing the pyrimidine dimer on the potential leading strand also became completely replicated, DpnI-treated DNA was treated with T4 endonuclease V and analyzed as described above for the lagging strand construct. Densitometric analysis of the relative amount of labeled Form I DNA indicated that -59% of the replicated Form I DNA is sensitive to nicking (Fig. 5B, compare third and fourth lanes). While this value is somewhat higher than the value obtained with the lagging strand construct (45% see above), in both cases, the value is close to 50%, which would be expected if a single round of replication occurred on both the undamaged and adducted strands. However, although small differences between the extent of replication of the damaged strand in these two constructs may be revealed by further analysis, the observation that the relative amount of T4 endonuclease V-sensitive Form I DNA (Fig. 5, A, third and fourth lanes; and B, third and fourth lanes) is close to 50% in both cases suggests that both the undamaged and dimer-containing strands are replicated in both orientations. The results obtained using these two constructs, which contain a single cyclobutane pyrimidine dimer in the same sequence context, but with a different orientation relative to the origin of replication, suggest that this lesion does not significantly block DNA replication when located on the lagging strand under these conditions in HeLa cell extracts in vitro. When the lesion is on the putative leading strand, a small reduction in replication efficiency was observed, which could suggest that this lesion has a more pronounced inhibitory effect on leading strand replication compared with replication on the lagging strand.

Effect of a Single (6-4) Photoproduct on DNA Replication in Vitro—To determine whether the mammalian replication complex responds differently to different types of UV-induced lesions, replication reactions were carried out using the pZ189-TT construct having a (6-4) photoproduct, instead of a cyclobutane pyrimidine dimer, at the TT site. In this analysis, the lesion was on the potential lagging strand template for DNA replication in both modified constructs. When the (6-4)-containing template was compared with the pyrimidine dimer-containing template and the unmodified template in the same experiment by measuring incorporation of radioactivity from \([\alpha\text{-}^{32}\text{P}]\text{dCTP} \] into trichloroacetic acid-precipitable material (17), the extent of inhibition of replication by the (6-4) photoproduct was greater than that obtained with the pyrimidine dimer-containing plasmid (Fig. 4). The extent of replication, as determined from the incorporation of radioactivity into trichloroacetic acid-precipitable material, was reduced to an average of \(-56 \pm 19\%\) of that of the unmodified control plasmid in seven separate experiments using three different preparations of (6-4) photoproduct-containing and control plasmids. Inhibition of replication in vitro was confirmed by purification of the product DNA from duplicate reactions performed using either the (6-4) photoproduct-containing plasmid or the unmodified control construct and analysis by agarose gel electrophoresis and autoradiography (Fig. 7A). In experiments in which the replicated DNA was treated with DpnI, the labeled DNA synthesized in the presence of SV40 large T antigen was resistant to digestion, indicating that this represents completely replicated DNA molecules, while labeled DNA from the minus-T antigen reaction was sensitive to DpnI digestion (Fig. 7B, right panel). While these experiments only examine the effect of this...
lesion on replication in vitro when it is located on the lagging strand template, they demonstrate that the replication complex responds differently to the two major UV-induced lesions in the template and that a (6-4) photoproduct is substantially more inhibitory to DNA replication when compared with a cyclobutane pyrimidine dimer in the same DNA sequence context.

Mutagenesis following in vitro replication of the specifically modified plasmids—Replicated DNA, from experiments with the lagging strand template only, was treated with DpnI and with dam methylase and used to transform the mutagenesis tester strain, E. coli MBM7070. The methylation step was tested in some experiments by redigestion of an aliquot of the methylated DNA with DpnI, followed by agarose gel electrophoresis and autoradiography; the remethylated DNA was sensitive to DpnI digestion (data not shown). Screening for base changes at the lesion site was carried out by hybridization analysis. This hybridization method has been previously shown to allow detection of all possible single-base substitutions, a tandem double-base substitution, and a single-base deletion at the TT site (20). Plates of transformants were therefore screened for the presence of colonies carrying mutations in the 19-mer insert sequence by hybridizing with the wild-type sequence and scoring colonies that failed to hybridize at 56°C. Control experiments, in which colonies transformed with plasmids having either the wild-type TT sequence or CT or CC at the modification site were included on each plate used for hybridization analysis, demonstrated that under these conditions, only the wild-type sequence hybridized with the labeled 19-mer (Fig. 8).

In the case of the plasmid containing the pyrimidine cyclobutane dimer on the lagging strand, 3046 transformants were screened in total from three experiments, and 138 nonhybridizing colonies (4.5%) were identified. Sequence analysis of plasmid DNA isolated from 22 of these colonies revealed that 21 of 22 (95%) of these plasmids were construction-related alterations, which lacked the 19-mer insert, contained more than one 19-mer insert, or had a base change in the AattI ligation site. The observed failure of plasmids containing multiple inserts to hybridize may possibly be due to the formation of a hairpin structure in this region during the washing process, which prevents the 19-mer from annealing. One plasmid, which did not belong to any of the above classes, contained an A → T transversion within the insert sequence, but that was not targeted to the lesion site. These data indicate that the frequency of targeted mutations is <0.2%, assuming that >95% of the total nonhybridizing colonies represent construction-related alterations. This construction-related background frequency is similar to that reported previously for the construction of single-stranded vectors using this specifically modified oligonucleotide (19–23). Plasmid DNA was also prepared from 40 randomly chosen vectors that gave a positive hybridization signal under these conditions. These plasmids all carried the wild-type 19-mer sequence (data not shown), making it unlikely that the screening process failed to detect plasmids carrying mutations at the lesion site.

In the case of the (6-4) photoproduct-containing plasmid, a total of 3050 colonies were screened, resulting in 88 nonhybridizing (2.9%) colonies. Sequence analysis of 45 colonies revealed that 41 of 45 (93%) belonged to the background categories listed above. Of the remaining four, one had a single-base deletion (TT → T) targeted to the lesion site. The other three comprised A → C and A → T transversions at position 4 in the 11-mer sequence (5'-GCAAGTTGGAG-3') and a deletion of G at position 5 in this sequence. Assuming that 91% of the nonhybridizing colonies were of the background type, the mutation frequency with this plasmid was <0.3%. These numbers of mutations are too low to make any conclusion regarding their specificity. However, it is possible to conclude that replication of the lesion-containing plasmids in vitro is relatively accurate. The percentage of nonhybridizing colonies observed with the replicated modified DNA was similar to that observed following transformation of the bacterial tester strain with DpnI-treated, unmethylated replicated plasmid (4 in 240 colonies; 1.6%), supporting the conclusion that the background is due to the construction and is not related to the insert modification. In addition, screening of transformants following direct transformation of the tester strain with these constructs, without prior incubation in the DNA replication reaction in vitro, also showed that the background of triple inserts, parent vector,
and AatI site mutations was already present in the construct preparation (data not shown). These results demonstrate that in vitro replication of plasmids containing either a single cyclobutane dimer or (6-4) photoproduct at a TT site occurs without the generation of a large number of mutations targeted to the lesion site.

These experiments do not address the mechanisms involved in mutagenic and nonmutagenic replication, for example whether gaps are left opposite the lesion or whether replication may be templated by the undamaged strand (see "Discussion"). The detection of misincorporations as mutations in the transformation assay is dependent on the base changes being present in closed circular or nicked DNA (30) prior to transformation of E. coli MBM7070. It is therefore possible that some misincorporation events, which are present in replicative intermediate DNA forms, for example, would not be detected by this assay.

DISCUSSION

To investigate the effect of UV-induced DNA damage on DNA replication and mutagenesis, we have constructed DNA templates containing a single TT cyclobutane dimer or (6-4) photoproduct and examined the efficiency with which these constructs are replicated in human cell extracts in vitro. These two lesions have substantially different effects on DNA replication in vitro. Plasmid DNA containing a cyclobutane dimer on the lagging strand template is replicated as efficiently as an unmodified plasmid in HeLa cell extracts in vitro. A small reduction in replication efficiency was observed when the dimer was on the leading strand, suggesting a potential difference in the effect of this lesion on leading compared with lagging strand replication. In contrast, the presence of a (6-4) photoproduct on the lagging strand template inhibits replication in vitro substantially, by ~50%. DpnI-resistant, completely closed circular product DNA from the replication of the dimer-containing plasmid is sensitive to nicking by T4 endonuclease V, indicating the presence of a dimer in the product DNA. It has been suggested that replication of UV-induced lesions may result in gaps in the newly synthesized strand at the sites of the lesions, which are subsequently filled in prior to ligation of the product DNA fragments (32). While the kinetics of replication in vitro suggest that the steps involved in the replication reaction are similar in the case of both the undamaged and lagging strand dimer constructs, a difference could only be observed if the proposed gap-filling step was the rate-limiting step under the conditions analyzed here. Given that experiments on the replication of undamaged SV40-based plasmids in vitro indicate that the rate-limiting step is initiation at the SV40 origin rather than the elongation phase (24, 37–39), it is unlikely that information on the mechanism of DNA synthesis on the damaged strand could be derived from the kinetics of incorporation of radioactivity alone. However, analysis of the kinetics of formation of the products of the DNA replication reaction, by agarose and polyacrylamide gel electrophoresis, has the potential to provide important information on the mechanism of replication of damaged DNA in this system. Recent experiments in which plasmids containing a single acetylaminofluorene adduct were replicated in vitro indicate that the undamaged strand may be preferentially replicated in these templates, providing a potentially error-free mechanism to overcome lesions in the template (33). It is possible that a comparable mechanism could play a role in the inhibition of replication observed in the presence of the (6-4) photoproduct. Information on the precise mechanism by which double-stranded DNA containing a UV-induced lesion is replicated by the complete replication complex will come from a more detailed analysis of the structure and sequence of nascent DNA formed during the DNA replication reaction in vitro.

Purified DNA polymerase I from E. coli has been shown to be capable of mutagenic bypass of a single cis,syn-cyclobutane pyrimidine dimer in vitro (34–36). The replication of plasmid DNA containing a single UV-induced lesion, described here, is consistent with the previous observation that plasmids containing randomly located UV-induced lesions can be replicated in mammalian cell extracts in vitro (17, 18). Thus, the cell extract contains the essential cellular proteins involved in this process. Whether proteins in addition to those known to be necessary and sufficient for replication of undamaged DNA in vitro (37–39) are required for replication of lesion-containing DNA can be elucidated using this system. In contrast to the present results using a complete replication system, DNA synthesis by purified DNA polymerase δ, which is essential for replication in vitro, is completely blocked by UV-induced lesions in the template (40). Another major replicative DNA polymerase, DNA polymerase α, can carry out synthesis past a pyrimidine dimer in vitro only in the presence of the accessory protein proliferating cell nuclear antigen (41). These observations suggest that the properties of the complete replication complex rather than those of the isolated polymerases may be important in determining the effect of UV-induced lesions on DNA replication.

The present experiments utilize extracts from nonirradiated HeLa cells. Extracts from UV-irradiated HeLa cells have reduced DNA replication activity toward both nonirradiated (10) and randomly UV-irradiated pZ189 DNAs in vitro, suggesting that replication activity following irradiation is controlled by...
mechanisms in addition to fork blockage. In mammalian cells in vivo, UV irradiation results in an arrest of DNA replication; however, UV-damaged cellular DNA ultimately becomes replicated, and 24 h after irradiation, newly replicated, double-stranded DNA contains numerous UV photoproducts in the parental strand (42). The observation that, in repair-deficient mammalian cells from xeroderma pigmentosum patients, UV-damaged DNA is replicated in the absence of DNA repair (4, 43) is also consistent with the conclusion that UV-induced lesions do not constitute an absolute block to DNA replication in vivo. The differential ability of the two major UV-induced lesions to inhibit DNA replication may have significance in the cell for the generation of signals for repair proteins or cell cycle arrest, although the mechanisms involved in the initial steps of these processes are not known. The interaction of the DNA replication machinery with RNA polymerase II transcription complexes, which are stalled at sites of DNA damage in the template (44, 45), could also be important in signaling repair to occur (46). The recent demonstration of inducible cellular processes through which replication can be arrested following exposure to DNA-damaging agents, by, for example, inhibition of processive DNA synthesis by RNA polymerase II through the action of the p21 protein (16), suggests that the replication arrest observed following UV irradiation may be in part attributable to these processes rather than solely to blockage of replication fork progression by lesions in the template. In addition, UV irradiation-induced phosphorylation of replication proteins, such as human single-stranded DNA-binding protein (10, 47), could play a role in the response of the replication complex to lesions in the genome of UV-irradiated cells. It is interesting to note that (6-4) photoproducts are much more efficiently repaired both in vivo (48–50) and in cell extracts (51) than are pyrimidine dimers, indicating that this lesion may be recognized differently by both the replication and the repair machinery in the cell.

The relative accuracy of replication of the TT dimer-containing plasmid is consistent with observations from a number of test systems and from studies of mutations occurring in human tumors in situ, which indicate that TT sites are under-represented in the spectrum of UV-induced mutations (reviewed in Refs. 4 and 52). UV-induced mutations tend to be predominantly at C-containing lesion sites. It has been proposed that the A-rule may account for the relative low mutagenicity of lesions at TT sites, such that the replicative polymerase preferentially inserts an A opposite a lesion site, leading to the correct base opposite a T in the lesion. However, evidence from studies on site-specific lesions in E. coli indicates that different lesions may have different capacities to code for the correct base, suggesting that the A-rule may not fully explain the observed lack of mutagenesis in all cases (19–23). In E. coli, replication of a single-stranded vector containing a single cis, syn-TT dimer in SOS-induced cells yields 7.6% targeted mutations (19). In contrast, the same lesion replicated in the yeast Saccharomyces cerevisiae yields only 0.4% targeted mutations (22, 23), indicating that the mutagenicity of this lesion can differ by almost 20-fold in different organisms. The (6-4) photoproduct has been shown to be more highly mutagenic than the cyclobutane dimer in bacteria, yielding 90% targeted mutations in SOS-induced cells, possibly due to its greater ability to distort the structure of the DNA backbone (21). In contrast, replication of single-stranded vectors containing a single (6-4) photoproduct in S. cerevisiae results in only 12–20% targeted mutations, possibly reflecting differences in the ability of yeast and E. coli DNA polymerases to copy this lesion (53). The apparent low mutagenicity of this lesion following replication in mammalian cell extracts in vitro could also reflect a difference between the responses of the bacterial and mammalian replication complexes to this lesion. However, several characteristics of replication in vitro must be considered in the interpretation of these results. It should be considered that in vitro, differences in the rate of elongation of molecules containing a misincorporation at the lesion site, such that these molecules are not processed into Form I DNA during the incubation period and so fail to transform the bacterial tester strain, could also influence the observed mutation frequency. In addition, in vitro replication of plasmids carrying certain site-specifically located single acetylaminofluorene adducts has shown that replication of the most mutagenic acetylaminofluorene adduct is ~99% accurate in vitro (33). In these experiments, the undamaged DNA strand was found to be preferentially replicated in the modified construct (33). In the absence of direct evidence that the (6-4) lesion has been copied, it is possible that the low mutagenicity of the (6-4) lesion observed in the present experiments is a consequence of replication of only the unmodified strand rather than representing an inherent characteristic of replication of this lesion. However, while these features of DNA replication in vitro may lead to a lower estimate of the mutagenicity of certain lesions, the system also provides a means to directly analyze the relationship between synthesis arrest, lesion bypass, and mutagenicity. In particular, direct analysis of the newly replicated nascent DNA around the lesion site for sequence changes may be possible, providing further insight into the process of mutation fixation.

The approach described should be useful in defining more completely the molecular basis of replication of UV-damaged templates and in characterizing the mutagenic potential of both UV- and chemical-induced lesions in the mammalian genome.

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