Bypass Replication in Vitro of UV-Induced Photoproducts Blocking Leading or Lagging Strand Synthesis†

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ABSTRACT: In vitro replication assays were used to determine the capacity of HeLa extracts to replicate past one of the two major photoproducts produced by ultraviolet radiation at adjacent thymines in duplex DNA, namely, the *cis,syn* cyclobutane dimer ([*c,s*]TT) and the 6-4 pyrimidine-pyrimidone adduct ([6-4]-TT). The site-specific photoproduct was placed on the template either to the leading strand or to the lagging strand of nascent DNA with respect to the first fork encountering the lesion during bidirectional replication of closed circular duplex molecules carrying the SV40 origin. Replication products from time-course reactions were fractionated by gel electrophoresis in the presence of ethidium bromide. Recognition and quantification of true translesion synthesis products, i.e., newly synthesized closed circular molecules carrying the photoproduct, were aided by specific substrate modifications (a T:T mismatch in a unique *Pst*I site nearby the photoproduct) and improved assay conditions (internal standard to control for completion of *Pst*I digestion). Extracts from HeLa cells, which express DNA polymerase η, were competent to replicate past the [*c,s*]TT on either strand. The efficiency of bypass replication of the [*c,s*]TT on the template to the leading or the lagging strand was 71% and 67%, respectively. The same extracts demonstrated very low efficiency of translesion synthesis (at most 8−10%) of the [6-4]TT on either template position. Replication-competent cell-free extracts from other human cells were also deficient in the bypass of the [6-4]TT in vitro.

Ultraviolet light (UV)[1] generates two major classes of photoproducts in DNA, the *cis,syn* cyclobutane pyrimidine dimer (CPD) and the 6-4 pyrimidine-pyrimidone adduct (6-4PP), as well as other minor photoproducts (*1*). These lesions lead to the transient inhibition of DNA synthesis due to passive effects (block of DNA elongation) and activation of cell cycle checkpoints (*2, 3*). The latter mechanism provides more time for nucleotide excision repair (NER) to remove photoproducts from DNA before it is replicated (*4*). Nonetheless, some induced photoproducts remain in DNA and interfere with replication. The inability of the principal DNA polymerases to complete the replication of DNA-containing photoproducts is circumvented by specialized bypass DNA polymerases that catalyze translesion synthesis (TLS). The low fidelity of replication by these bypass polymerases is probably responsible for the bulk of UV-induced mutagenesis.

The bypass polymerases implicated so far in TLS of photoproducts in eukaryotes are polζ, polη, and polι (reviewed in *5*−*7*). Genetic and biochemical studies in *Saccharomyces cerevisiae* identified the Rev3p/Rev7p complex as the error-prone polζ. Along with Rev1p, polζ is required for the bypass of the 6-4 pyrimidine-pyrimidone thymine adduct ([6-4]TT), but not the *cis,syn* cyclobutane thymine dimer ([*c,s*]TT) (*8*), even though this enzyme was shown to replicate past the [*c,s*]TT in vitro with an efficiency of approximately 10% (*9*). It turns out that yeast polζ is most adept at extending from nucleotides inserted opposite the 3′T of either photoproduct by other DNA polymerases (*10, 11*). Reduction of polζ and Rev1p in human cells expressing specific antisense RNAs decreased UV-induced mutagenesis significantly (*12, 13*). Polη is a bypass polymerase capable of replicating past [*c,s*]TT efficiently and accurately (*14*−*17*), but it is unable to bypass the [6-4]TT (*14, 17*). Polη can insert one nucleotide opposite the 3′T of [6-4]TT, and by doing so it might aid in the bypass of this lesion by polζ (*11, 18*). Polη reduces significantly the mutagenic and carcinogenic effects of UV in human cells. Absence of polη causes the cancer-prone hereditary disease xeroderma pigmentosum variant (XP-V). Cells derived from XP-V patients carry mutations in the gene encoding polη (*19*−*21*), and their

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[1] Abbreviations: [6-4]PP, 6-4 pyrimidine-pyrimidone adduct; [6-4]-TT, 6-4 pyrimidine-pyrimidone adduct at adjacent thymines; CPD, cyclobutane pyrimidine dimer; [*c,s*]TT, *cis,syn* cyclobutane thymine dimer; dNTPs, deoxynucleotide triphosphates; Klenow, fragment of *E. coli* DNA polymerase I carrying only the catalytic site for DNA polymerization; MMR, mismatch repair; NER, nucleotide excision repair; nt, nucleotide(s); oriL and oriR, positions of the SV40 origin of replication to the left or the right of the lacZα gene in M13mp2SV, in which the site-specific photoproduct was inserted; pol, DNA polymerase; Tag, SV40 large T antigen; TLS, translesion synthesis; UV, ultraviolet light; XP, xeroderma pigmentosum; XP-V, xeroderma pigmentosum variant; #-mer(s), oligonucleotide(s) composed of the indicated number (#) of units.

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extracts are defective in bypass replication of [*c,s*]TT (*22*−*25*). Conversely, pol*ι* has the ability to insert nucleotides opposite the [6-4]TT much more efficiently than opposite the [*c,s*]TT (*18, 26*). It is presumed that the incorporated nucleotides might be extended in vivo by either pol*ι* (*18*) or pol*ζ* (*10*).

The finding that the majority of UV-induced mutations in humans consist of 3′C→T transitions in both NER-proficient (*27*) and -deficient cells (*28, 29*) suggests that this type of mutation originates primarily from a 3′C-containing CPD. This inference is likely to be correct because CPDs are the most abundant photoproducts induced by UV (*1*) and [6-4]-PPs are repaired at faster rates than CPDs (*30*). NER in coordination with DNA damage-induced cell cycle delays should eliminate most [6-4]PPs from DNA prior to replication. Genetic studies in yeast suggested that in addition to bypassing [*c,s*]TT accurately, pol*η* also has a role in error-free bypass of 3′CT and 3′CC cyclobutane dimers (*31*). Thus, most 3′C→T transitions at CPDs must arise at those sites in which pol*η* failed to bypass the lesion accurately and A was incorporated opposite the 3′C and extended by pol*ζ* (*18, 31*). Interestingly, the absence of pol*η* in XP-V cells leads not only to substantial increases in UV-induced mutations but also to significant changes in the spectrum and distribution relative to the two strands of duplex DNA (*27*). In vitro studies (*25*) have corroborated in vivo observations (*27*) that the predominant UV-induced base substitution involving cytosines in XP-V is a C→A transversion, all arising from photoproducts located in one strand. The in vitro experiments afforded the identification of this location as being the template to the leading strand (*25*). It has been suggested that replication past CPDs in the absence of pol*η* depends on the participation of pol*ι*, either by itself or in combination with pol*ζ* (*18*). Favored incorporation of dTTP opposite C or T of a CPD by pol*ι* would explain the higher frequencies of C→A and T→A transversions in XP-V (*32*), but not the bias for C→A transversions when the photoproduct is on the template to the leading strand (*25*).

It is conceivable that bypass replication during leading and lagging strand synthesis relies on different mechanisms. Roberts and collaborators have found that the fidelity of replication of undamaged DNA by a HeLa extract was lower during lagging strand synthesis than during leading strand synthesis (*33*). On UV-irradiated templates, the average probability for C→T transitions was found to be the same in both strands (*34*). However, the distribution of this type of substitution was very different for leading and lagging strand replication. Some sites yielded more errors during lagging strand synthesis than during leading strand synthesis, while for other sites the reverse relationship was observed (*34*). Psoralen monoadducts (*35*) and acetylaminofluorene adducts (*36*) were bypassed with lower fidelity during in vitro replication when these lesions were on the lagging strand template. Bypass replication of these two types of DNA lesion is also dependent on pol*η* (*37, 38*).

The mutagenic properties of [6-4]TT were characterized in vivo using circular vectors containing a single photoproduct and transfected into bacteria, yeast, and mammalian cells (*39*−*43*). The [6-4]TT was highly mutagenic in SOS-induced bacteria, generating 3′T→C transitions in the majority of the replicated molecules (*39, 40*). In contrast, [6-4]TT in single-stranded or gapped duplex vectors was less mutagenic in yeast, where 60−70% of molecules replicated accurately and 12−20% displayed 3′T→C substitutions (*41*). Replication in simian COS7 cells of a site-specific [6-4]TT on a single-stranded vector induced a mutation frequency of 60%, but the most common substitution was a semi-targeted G→T transversion (*42*). In the same DNA repair-proficient background (COS7 cells), the replication of double-stranded closed circular molecules carrying a single [6-4]TT induced mutation at lower frequencies, but they were primarily targeted transitions (50−60% 3′T→C) (*43*). Other interesting findings were that the [6-4]TT was more mutagenic than [*c,s*]-TT and that both lesions were 2−3 times more likely to induce a mutation when bypassed during lagging strand synthesis (*43*). In contrast to these observations, Carty and collaborators (*44*) reported that the [6-4]TT on the template to the lagging strand did not elicit mutations in daughter molecules generated during in vitro replication with a HeLa extract.

Further evidence that the [6-4]TT is mutagenic in human cells arose from studies of the frequency and spectrum of mutations in UV-irradiated shuttle vectors transfected into NER-deficient cells (XP-A). Otoshi and collaborators (*45*) used in vitro photoreactivation catalyzed by the CPD or the (6-4) photolyase to remove each type of photoproduct from irradiated DNA prior to transfection. Replication of [6-4]-PP-containing closed circular molecules caused predominantly C→T transitions (63% of all point mutations). However, 14% of all transitions observed were T→C mutations, and most of them occurred at TT sites. Transition mutations at TT sites were not detected after [6-4]PP removal, consistent with the bypass replication of [*c,s*]TT being done efficiently and accurately by pol*η*.

Taken together, these observations suggest that the fidelity of bypass replication depends on the structure of the DNA lesion, the nucleotide sequence surrounding it, the properties of the bypass DNA polymerases, and also on the location of the DNA lesion relative to the displacement of the replication fork. We constructed closed circular heteroduplexes containing either a [6-4]TT or a [*c,s*]TT on the template to the leading or the lagging strand to use as substrates for in vitro replication by human cell extracts. Results obtained in this study demonstrated that extracts from human cells display very low capability for bypass of the [6-4]TT on either template strand.

EXPERIMENTAL PROCEDURES

Materials. M13mp2SV (oriL and oriR) and the human colorectal cancer cell line HCT116 (hMLH1 mutant, MMR defective) were obtained from Dr. Thomas A. Kunkel (National Institute of Environmental Health Science, Research Triangle Park, NC). HeLa S3 cells were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Facility (University of North Carolina at Chapel Hill) and from the National Cell Culture Center (Minneapolis, MN). SV40-transformed XP-A cells (XP12BE, NER deficient) were obtained from the NIGMS Human Genetic Mutant Cell Repository (GM4429). XP-V fibroblasts (deficient in pol*η*) were derived from XP4BE by transformation with the SV40 large T antigen gene (*46*). T4 DNA polymerase and DNA ligase were purchased from Roche Molecular Biochemicals. T4 polynucleotide kinase, *Pst*I and *Bsr*FI restriction enzymes,

and DNA polymerase I (Klenow fragment) were purchased from New England Biolabs. T4 endonuclease V was a gift from Dr. Stephen Lloyd (University of Texas Medical Branch at Galveston). The supplier of purified SV40 large T antigen was Molecular Biology Resources, Inc. (Milwaukee, WI). The Pathology Oligonucleotide Synthesis Facility (University of North Carolina at Chapel Hill) synthesized the oligonucleotides used in this study.

Preparation and Characterization of 40-mers Containing a Single [c,s]TT or [6-4]TT Photoproduct. Two micromoles of the 8-mer 5′GTATTATG was dissolved in 10 mL of 10 mM Tris (pH 8), 1 mM EDTA. This solution was placed in a Petri dish on ice and irradiated with ~2 mW of 254 nm light for 2 h (with mixing at 15 min intervals). Oligomers containing one of the two major UV-induced photoproducts ([*c,s*]TT or [6-4]TT) were fractionated and repurified according to published procedures (*47*). HPLC analyses showed that the purified oligonucleotides carrying a single photoproduct were not contaminated with undamaged oligonucleotides. The 8-mers were ligated to a 15-mer and a 17-mer with the help of a 29-mer scaffold. The sequence of the resulting 40-mers: 5′GTTATCCGCTCACTGC**T**GTA**XX**-ATGTCTAGTCACAATTCC, included a point mutation (boldface underlined T) in the *Pst*I recognition sequence (underlined) and a photoproduct in the **XX** position. After purification by electrophoresis in 15% polyacrylamide−7 M urea gels, the identity of the inserted photoproduct and the purity of the modified 40-mers were confirmed. An aliquot of each 40-mer was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, according to manufacturers' instructions (New England Biolabs). The labeled 40-mers were annealed back to the scaffold, and the double-stranded oligomers were treated with T4 endonuclease V, as previously described (*22*). In separate reactions, the labeled 40-mers were treated with hot piperidine (95 °C) for 5, 25, or 120 min (*48*). Primer extension reactions were carried out after annealing each 40-mer template (10 pmol) with 1.5 pmol of 5′ ^{32}P-labeled primer (5′GGAATTGTGACTA) in 10 μL of reaction buffer (New England Biolabs) for the Klenow fragment of *E. coli* DNA polymerase I. After addition of 1 unit of Klenow and 100 μM dNTPs, the reactions were incubated at 37 °C for 20 min and stopped with 20 μL of 95% formamide, and primer-extension products were fractionated by electrophoresis in 15% polyacrylamide−7 M urea gels. The dried gels were exposed to a phosphor screen, which was scanned with a Storm 840 PhosphorImager (Molecular Dynamics, Inc.).

Construction of Double-Stranded, Closed Circular Heteroduplexes Containing a Single [c,s]TT or [6-4]TT. The sequence 5′ACTAGACATAATACTGCAGTC was inserted into the lacZα gene of M13mp2SV oriL and M13mp2SV oriR (*49, 50*) by site-directed mutagenesis (*51, 52*). This insertion introduced a single *Pst*I site (underlined) into the new viral constructs (oriL-Pst and oriR-Pst). Closed circular, single-stranded oriL-Pst or oriR-Pst (+ strands) were purified and annealed with 40-mers containing a point mutation in the *Pst*I recognition site (T replacing A) and a single [*c,s*]-TT or [6-4]TT photoproduct (or undamaged di-thymines). Second-strand synthesis, ligation, and purification in CsCl density gradients were done according to published procedures (*22, 53*).

Replication of Heteroduplex Molecules in Vitro and Product Analysis. Cell-free extracts were prepared according to published protocols (*54, 55*). In vitro replication reactions in the presence of SV40 large T antigen (Tag) were carried out in 25 μL reactions, as described (*22*), except for the inclusion of a preincubation step (*56*). Closed circular heteroduplex molecules were first incubated with Tag and extract for 20 min at 37 °C in the presence of all other reaction components, except dNTP precursors. [α-^{32}P]dCTP and cold dNTPs were added (time zero) and the incubations extended for the indicated periods of time. In vitro replication reactions in the absence of Tag were carried out in parallel. Addition of an equal volume of a solution containing 2% SDS, 2 mg/mL proteinase K, and 50 mM EDTA was used to stop the reactions. Then, equal amounts of an internal standard were added to the reactions prior to DNA purification using the QIAEX II Gel Extraction System (Qiagen). The internal standard was pUC19 (2686 bp) linearized with *Bsr*FI (leaves the single *Pst*I site at the center of the linear molecule) and labeled at the ends with [α-^{32}P]dCTP and Klenow. This labeled standard helped monitor DNA recovery and whether *Pst*I digestion of replication products was complete in each of the replication reactions. Half of the purified DNA was digested with 60 units of *Pst*I at 37 °C for 3 h. Electrophoresis of the digested and undigested samples was carried out in a 1% agarose gel in the presence of 0.2 μg/mL ethidium bromide. Dried gels were exposed to a phosphor screen and scanned with the Storm 840 PhosphorImager.

The signal intensity for specific DNA bands was determined from the pixel volume (corrected for background), as quantified by the ImageQuaNT software (Molecular Dynamics, Inc.). The relative amount of each DNA species was normalized to the amount of pUC19 in each lane. The contribution of each parental strand in the formation of completely replicated, closed circular molecules (Form IV) was determined from the normalized amount of Form IV DNA, before and after *Pst*I digestion. The total amount of Form IV produced in a given reaction was determined in the samples not treated with *Pst*I. The amount of resistant Form IV DNA (product of the strand carrying the mismatch and the photoproduct) was that remaining after *Pst*I treatment. Form IV molecules sensitive to *Pst*I digestion (replication products of the undamaged strand) were converted to linear molecules (Form III). Nicked or gapped circular molecules (Form II) were also quantified. If necessary, the fraction of full-length pUC19 molecules remaining in *Pst*I-digested samples (ratio of pixel volume in full-length pUC19 band over total pUC19, i.e., sum of pixel volumes in bands corresponding to digested and undigested pUC19) was used to correct the fraction of *Pst*I-resistant Form IV. The amount of radiolabeled pUC19 recovered in samples treated with *Pst*I was consistently less than expected from that present in the aliquot of the same sample not treated with *Pst*I. This finding appeared related to minor contamination of *Pst*I preparations with exonucleases capable of removing label from the end of the linear pUC19 molecules. Accordingly, the ratio of total radioactivity associated with all forms of replication products to the radioactivity in end-labeled pUC19 (internal standard) was higher in the *Pst*I-digested sample than in the aliquot of DNA purified from the same reaction, but not treated with the restriction enzyme. Therefore, the

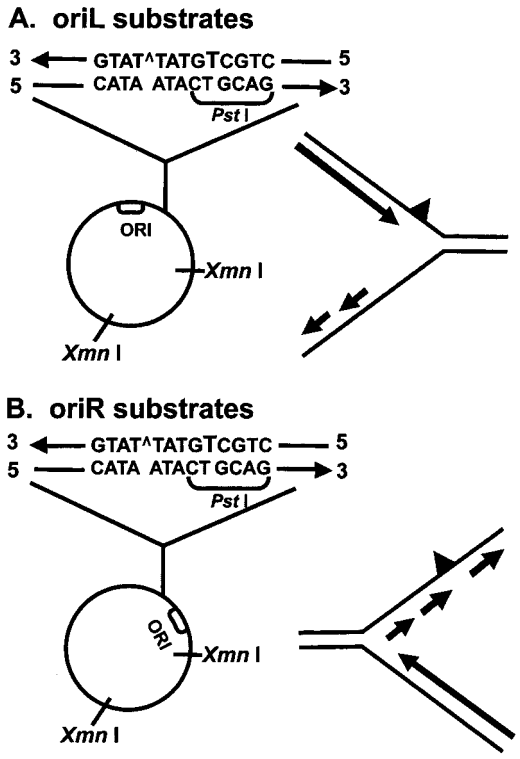

FIGURE 1: Double-stranded circular heteroduplexes used as substrates for bypass replication assays. A site-specific photoproduct was placed on the template for the leading (oriL) or the lagging strand (oriR). Three oriL and three oriR constructs were prepared, each containing a T:T mismatch in the recognition site for *Pst*I and a [6-4]TT, a [*c*,*s*]TT, or undamaged di-thymines at the position denoted by T∧T. The location of the SV40 origin of replication to the left (oriL) or to the right (oriR) of the photoproduct is indicated as ORI. The recognition sites for *Xmn*I are shown for orientation purpose only.

amount of pUC19 in the *Pst*I-treated samples (*P*2) was determined from the equation: $T1/P1 = T2/P2$. *T*1 was the total radioactivity measured in all forms of replication products in samples not treated with *Pst*I, *P*1 was the radioactivity in full-length pUC19 in the same sample, and *T*2 was the total radioactivity in replication products digested with *Pst*I. This corrected pUC19 value was then used to normalize the amount of *Pst*I-resistant Form IV.

RESULTS

Strand-Specific Templates for Measuring Bypass Replication of UV-Induced Photoproducts in DNA. The diagrams in Figure 1 illustrate the synthetic substrates used in this study to evaluate the capacity of HeLa extracts for replicating past photoproducts during leading and lagging strand synthesis. The oriL molecules contain the SV40 origin of replication to the left of the site-specific photoproduct, mainly a [*c*,*s*]-TT or a [6-4]TT. The distance from the center of the origin to the photoproduct is 409 nt. As DNA replication initiates at the origin and proceeds bidirectionally, the strand carrying the photoproduct serves as the template for leading strand synthesis by the first fork encountering the lesion. In the oriR molecules, the center of the SV40 origin of replication is found 392 nt to the right of the photoproduct. The first replication fork approaching the lesion uses the strand containing a [*c*,*s*]TT or a [6-4]TT as the template to the lagging strand of nascent DNA. Note that the fourth base pair on the 5′ side of the photoproduct is a T:T mismatch within a unique *Pst*I recognition site. This mismatch was included in all four constructs containing a UV-induced lesion and the two control molecules without any photoproduct. Upon in vitro replication, the product of the 3′→5′ strand in these six constructs will contain a mutated recognition site for *Pst*I. Therefore, it will be resistant to digestion by this restriction enzyme. Replication of the 5′→3′ strand, however, will reconstitute the correct recognition sequence for *Pst*I and the final product will be linearized upon treatment with the enzyme. Addition of this mismatch allows the identification of true bypass products of semi-conservative replication of a lesion-containing heteroduplex. It provides the means to distinguish between the daughter molecule generated from the strand carrying the photoproduct from that generated from the undamaged strand, thus increasing the specificity of the bypass replication assay (*23*). Considering the analytical importance of the *Pst*I digestion for the correct interpretation of results of in vitro bypass replication, an internal control for the efficiency of this reaction was included in each experiment. The end-labeled pUC19 internal standard, previously digested with *Bsr*FI, carries its single *Pst*I site at the center of the linear molecule. Digestion of this internal standard with *Pst*I results in two fragments that differ in length by only one nt. Thus, the efficiency of *Pst*I digestion of DNA from each single reaction could be directly assessed by whether full-length pUC19 was detected in the individual gel lanes (see below). If present, the small fraction of full-length pUC19 remaining after *Pst*I digestion was used to correct the estimated amount of the bypass product, i.e., the fraction of closed circular duplex molecules (Form IV) resistant to *Pst*I.

Judging from HPLC chromatographic profiles (*47*), the photoproduct-containing 8-mers used in this study were not contaminated with undamaged oligonucleotides before they were ligated into 40-mers carrying the mutated *Pst*I site. Then, the 40-mers containing a [6-4]TT, a [*c*,*s*]TT, or no photoproduct were treated with hot piperidine (*48*). Only the 40-mers carrying the [6-4]TT were cleaved at the photoproduct; the others remained full-length even after 2 h incubation in 1 M piperidine at 95 °C. After being annealed to complementary sequences and treatment with T4 endonuclease V, only the duplexes containing the [*c*,*s*]TT were cut by this enzyme. These assays indicated that the oligomer preparations containing the [6-4]TT, the [*c*,*s*]TT, or undamaged thymines were not cross-contaminated. Primer extension reactions with Klenow, which cannot bypass the [6-4]TT or the [*c*,*s*]TT (*57*), were carried out with a 5′ end-labeled primer (13-mer) annealed to the test 40-mers. Results in Figure 2 revealed that primer extension stopped before the lesion on both [6-4]TT- and [*c*,*s*]TT-containing templates. The absence of full-length product (end-labeled 40-mer) confirmed that the preparations of photoproduct-containing 40-mers were not contaminated with undamaged oligonucleotides.

In Vitro Replication of Substrates Containing a [6-4]TT or a [c,s]TT on the Leading Strand Template. Figure 3 illustrates the fractionation by gel electrophoresis of products of in vitro replication of oriL constructs by a HeLa extract. Substrates were preincubated for 20 min with Tag, cell extract, and the other reaction components, except dNTPs. The indicated incubation times (0.5−2 h) started with the

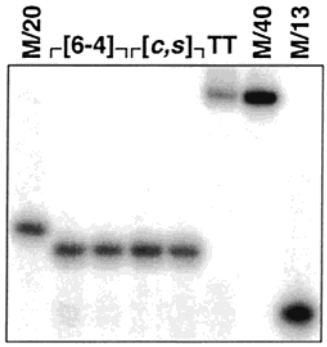

FIGURE 2: Primer extension by Klenow on 40-mer templates. The primer was a ^{32}P-5′-labeled 13-mer, and the templates were 40-mers containing a [6-4]TT, a [c,s]TT, or undamaged TT at positions 19 and 20 from the 3′ end. These 40-mers were assembled for incorporation into the double-stranded circular DNA heteroduplexes shown in Figure 1. M/13, M/20, and M/40 correspond to end-labeled oligonucleotides used as size markers. Only the primers annealed to the undamaged 40-mer were fully extended. When the template contained a photoproduct, extension was blocked at the lesion.

addition of DNA precursors. This two-step protocol reduced the lagging period usually observed when all reaction components are added together (*56*). Reaction mixtures lacking only Tag were incubated for 140 min to detect DNA synthesis activities other than origin-dependent semi-conservative DNA replication. Note that neither the TT-oriL control nor the photoproduct-containing substrates elicited significant incorporation of [^{32}P]dCMP into Form IV molecules in the absence of Tag (Figure 3, lanes 5).

The amount of radiolabeled Form IV, normalized to the amount of full-length pUC19 (internal standard) detected in the same gel lane, increased with time of incubation in the presence of Tag for all three substrates (Figure 4A−C). Upon digestion of the DNA replication products with *Pst*I, very little full-length pUC19 remained in the mixture. Under these conditions, the amount of radiolabeled Form IV replicated from TT-oriL (Figures 3A and 4A) was reduced to about 50% with a parallel increase in linear molecules (Form III). In the case of [*c*,*s*]TT-oriL, a substantial fraction of the radiolabeled Form IV was resistant to digestion by *Pst*I (Figures 3B and 4B), reflecting the capability of the HeLa extract to replicate past the cyclobutane dimer. Note, however, that the yield of radiolabeled Form IV from [6-4]-TT-oriL was much lower (Figures 3C and 4C). Furthermore, the Form IV synthesized in these reactions represented primarily the product of replication of the strand without the photoproduct. Only 5% or less of the Form IV generated from the [6-4]TT-oriL construct was resistant to digestion by *Pst*I in this experiment. Careful analysis of the efficiency of digestion of pUC19 by *Pst*I in several experiments showed that approximately 1−5% of the pUC19 internal control molecules escaped digestion.

The relative amounts of bypass product (fraction of Form IV resistant to *Pst*I digestion) observed in three independent experiments with the oriL constructs are summarized in Table 1. Given the semi-conservative nature of DNA replication, the expected fraction of products generated from each strand of the heteroduplex DNA is 0.5. Therefore, bypass replication efficiency can be defined as the ratio between the observed and the theoretical (0.5) fractions of *Pst*I-resistant Form IV. Normalized to the results obtained with the control TT-oriL construct, carrying the mutated *Pst*I site but no photoproduct, the results compiled in Table 1 show that the [*c*,*s*]TT on the leading strand template was bypassed in 71% of the molecules, compared to at most 10% for the [6-4]TT. Considering the error involved in determining correctly the minute amounts of *Pst*I-resistant Form IV generated from the [6-4]TT-oriL construct, these results suggest that the in vitro bypass replication of the [6-4]TT by HeLa extracts is very inefficient, if it occurs at all. Incubation of in vitro reactions for longer periods (4−6 h) did not increase significantly the amount of *Pst*I-resistant Form IV over that observed after 2 h (results not shown). Instead, long incubations seemed to favor the re-duplication of duplex molecules generated from the undamaged strand and the breakdown of fragile intermediates of DNA replication (*58*, *59*).

In Vitro Replication of Substrates Containing a [6-4]TT or a [c,s]TT on the Lagging Strand Template. The replication of oriR molecules by a HeLa extract is illustrated in Figure 5. These results, as well as those from longer periods of incubation (not shown), indicated that the [*c*,*s*]TT was

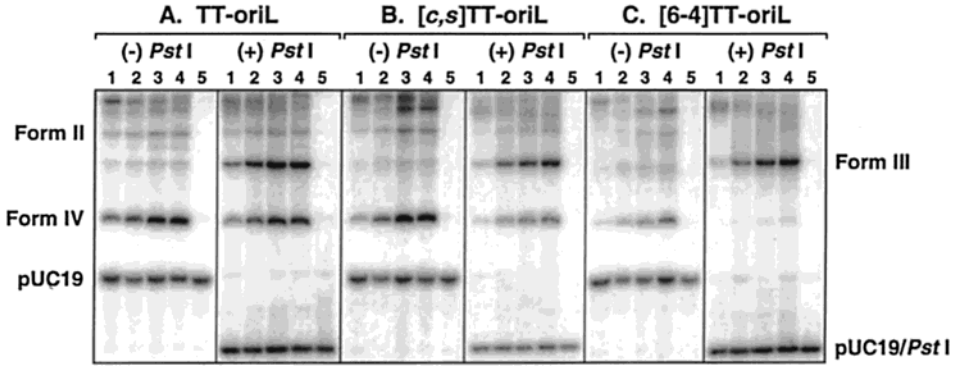

FIGURE 3: Time course of in vitro replication of oriL constructs by a HeLa extract. Reactions contained either the control molecule TT-oriL lacking a photoproduct (A) or the heteroduplexes carrying a site-specific [*c*,*s*]TT (B) or a [6-4]TT (C) on the template to the leading strand. Reaction mixtures containing all components, except dNTPs, were preincubated for 20 min at 37 °C. Following the addition of [^{32}P]dCTP and dNTPs, the reactions were incubated for 0.5, 1, 1.5, and 2 h (lanes 1−4, respectively). Reactions containing all components, except SV40 Tag, were incubated for 2 h and 20 min (lanes 5). Equal amounts of radiolabeled linear pUC19 were added to the reactions at the end of the incubation period and prior to DNA purification. Half of each sample was treated with *Pst*I. Undigested [(−)*Pst*I] and digested [(+)*Pst*I] samples were fractionated by electrophoresis in 1% agarose gels. The positions of replication Form II (nicked or gapped circular molecules), Form III (linear molecules), Form IV (closed circular molecules), full-length pUC19, and *Pst*I-digested pUC19 are indicated.

Table 1: Efficiency of Bypass Replication of the [*c,s*]TT and the [6-4]TT on the Template to the Leading or the Lagging Strand by HeLa Extracts

construct	photoproduct	relative amount of bypass product (*A*)[a]	bypass efficiency (*A*/0.5)[b]	normalized to non-dimer construct[c] (%)
OriL	none (TT)	0.49 ± 0.04 (*n* = 3)	0.98 ± 0.08 (*n* = 3)	100
	[*c,s*]TT	0.35 ± 0.08 (*n* = 3)	0.70 ± 0.16 (*n* = 3)	71
	[6-4]TT	0.05 ± 0.02 (*n* = 3)	0.10 ± 0.04 (*n* = 3)	10
OriR	none (TT)	0.44 ± 0.04 (*n* = 4)	0.87 ± 0.08 (*n* = 4)	100
	[*c,s*]TT	0.29 ± 0.08 (*n* = 4)	0.58 ± 0.16 (*n* = 4)	67
	[6-4]TT	0.03 ± 0.02 (*n* = 4)	0.07 ± 0.03 (*n* = 4)	8

[a] Relative amount of bypass product (*A*), measured as the fraction of Form IV resistant to *Pst*I digestion. Results of three or four independent experiments are shown. In vitro replication reactions were incubated for 1−2 h. [b] The maximum fraction of *Pst*I-resistant Form IV (product of the mismatch/photoproduct-containing strand) that can be generated during semi-conservative DNA replication of the heteroduplexes used in these experiments (Figure 1) is 0.5. Therefore, the ratio *A* over 0.5 defines the efficiency of bypass. [c] The efficiency of photoproduct bypass was divided by the efficiency of replication observed with the control construct, which carried the mismatch in the *Pst*I recognition sequence but no photoproduct at the di-thymines.

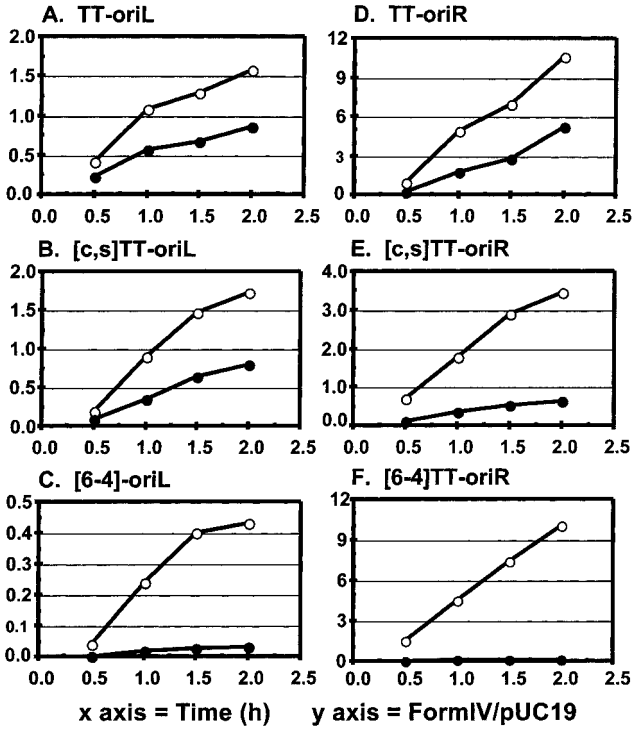

FIGURE 4: Graphic representation of the kinetics of synthesis of Form IV molecules by a HeLa extract. The total amount of Form IV detected in samples not treated with *Pst*I (open circles) and the amount of Form IV resistant to *Pst*I digestion (closed circles) (Figures 3 and 5) were normalized to the amount of pUC19 in each gel lane, as described under Experimental Procedures. Plotted against time of incubation at 37 °C were the Form IV/pUC19 values determined in replication products from control molecules without a photoproduct (A, D) or those containing a [*c,s*]TT (B, E) or a [6-4]TT (C, F) on the template to the leading strand (oriL, A−C) or the lagging strand (oriR, D−F). The relevant information in these panels is the time-course of Form IV synthesis and the relationships between the values for total (open circles) and *Pst*I-resistant (closed circles) Form IV/pUC19 in each panel.

bypassed in a fraction of replicated molecules. In contrast, very little replication past the [6-4]TT was evident in these experiments. Upon *Pst*I treatment, 41% of the Form IV synthesized from the control molecule (TT-oriR) was resistant to digestion, compared to 19% for the [*c,s*]TT-oriR construct. However, very little bypass product (~1% *Pst*I-resistant Form IV) was detected when the substrate was the [6-4]TT-oriR (Figure 4D−F). Other experiments yielded slightly better bypass replication results, as indicated by the average data compiled in Table 1.

One distinct feature of the replication of oriR molecules carrying a photoproduct was the accumulation of Form II. These are expected to be molecules in which a daughter strand gap was left opposite the dimer as the replication fork was displaced beyond this lesion. These Form II intermediates were readily observed among products of replication of the oriR constructs, but significantly less with the oriL constructs (compare Figures 3 and 5). This conclusion was also confirmed by results of bypass replication experiments using extracts from other human cells.

In Vitro Replication of oriL and oriR Constructs by Different Extracts. The capacity for bypass of the [6-4]TT lesion was also examined in extracts from human cell lines proven to be deficient in NER (XPA), MMR (HCT116), or polη-dependent bypass of [*c,s*]TT (XP-V). While displaying bypass of the [*c,s*]TT (except for the XP-V extract), none of these extracts showed much better capacity to bypass the [6-4]TT on the leading or lagging strand template than the HeLa extracts used in the experiments described above. OriR molecules carrying the photoproduct on the template to the lagging strand generated Form II molecules (gapped intermediates) in excess to that observed with the oriL molecules in all extracts. Figure 6 illustrates these observations for the HeLa and XP-V extracts. Note that even in the absence of polη and no capability for replicating past the [*c,s*]TT, the XP-V extract accumulated Form II intermediates among replication intermediates from oriR in greater excess than from oriL constructs.

DISCUSSION

The goal of this work was to compare the efficiency of bypass of the two major premutagenic UV-induced lesions, [6-4]TT and [*c,s*]TT, in double-stranded closed circular molecules during in vitro replication with cell-free extracts. We constructed substrates containing one or the other photoproduct in the same sequence context and approximately at the same distance from the SV40 origin of replication. Furthermore, the damaged sequence was placed at either side of the SV40 origin of replication, so that the photoproduct would be encountered during synthesis of the leading or the lagging strand of nascent DNA (Figure 1). A mismatch (T:T) was introduced in a single *Pst*I recognition

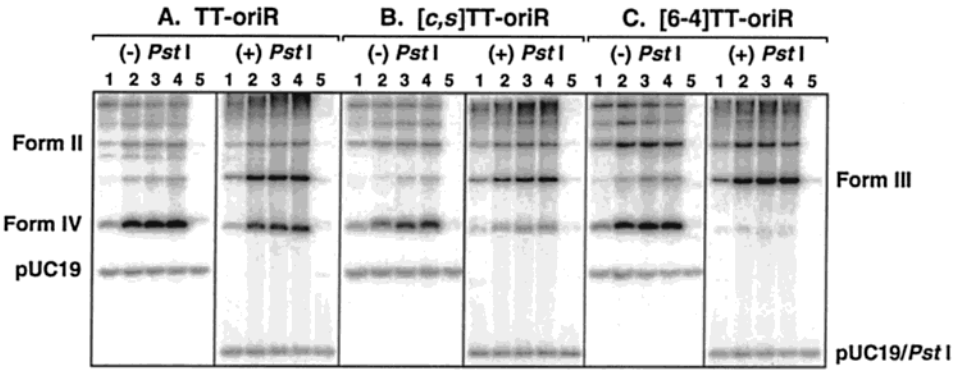

FIGURE 5: Time-course of in vitro replication of oriR constructs by a HeLa extract. Experimental conditions and illustration labels are as described in the legend to Figure 3, except that oriR constructs carrying undamaged di-thymidines (A), the [*c*,*s*]TT (B), or the [6-4]TT on the template to the lagging strand were used.

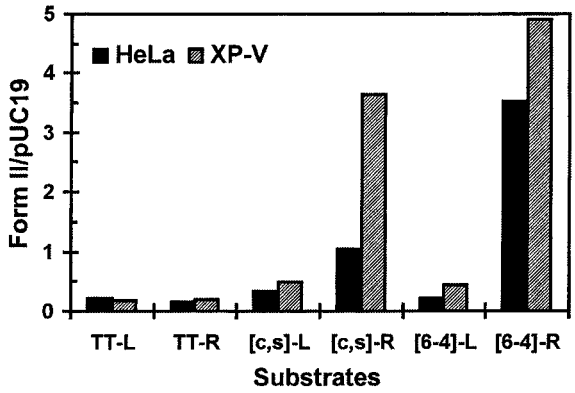

FIGURE 6: Gapped molecules (Form II) generated during in vitro replication of oriL and oriR constructs by extracts from HeLa (filled bars) or XP-V cells that lack polη (hatched bars). In vitro reactions were incubated for 2 h. The amount of Form II (gapped molecules) was quantified in *Pst*I-treated samples and normalized to the amount of the internal standard (pUC19). The six constructs are identified in the bar graph as follows: TT-L, TT-oriL; TT-R, TT-oriR; [c,s]-L, [*c*,*s*]TT-oriL; [c,s]-R, [*c*,*s*]TT-oriR; [6-4]-L, [6-4]TT-oriL; [6-4]-R, [6-4]TT-oriR.

site nearby the lesion to provide for the unambiguous recognition of bypass replication products. The mismatch rendered the input substrate and the duplex generated from the mismatch/photoproduct-containing strand resistant to digestion by *Pst*I. The daughter duplex generated from the undamaged strand, however, inherited the wild-type *Pst*I site and was sensitive to digestion by this restriction enzyme. In our experience, restriction digestion of DNA replication products purified from reaction mixtures with cellular proteins, using either extraction with organic solvents or commercially available DNA extraction systems, often does not go to completion. Therefore, the inclusion of an internal standard to evaluate the quality of the *Pst*I digestion was essential to the correct interpretation of bypass replication results (Figures 3−5). The use of parallel in vitro replication reactions containing *Pst*I-sensitive DNA was not as reliable and even led to misinterpretation of results in early experiments. However, the data obtained after the inclusion of the internal pUC19/*Pst*I standard demonstrated that the small fraction of Form IV DNA produced from [6-4]TT-containing constructs and surviving *Pst*I digestion (Figures 3 and 5) was close to the fraction of full-length pUC19 detected in the same sample. Together, the results presented here (Figures 3−5, Table 1) indicate that during SV40 origin-dependent in vitro replication of circular duplex DNA by HeLa extracts (contain polη), under conditions in which bypass replication of the [*c*,*s*]TT was readily observed, replication past the [6-4]-TT was barely detected.

Previous studies in this laboratory confirmed by two-dimensional gel electrophoresis (*22*) and electron microscopy (*59*) that circular duplex molecules are replicated bi-directionally from the SV40 origin of replication (*58*). All of our observations to date support the conclusion that a photoproduct constitutes a strong block to leading strand synthesis, but not to replication fork displacement. This latter finding is in line with the results of Veaute et al. (*60*), which showed that photoproducts in DNA do not inhibit the activity of the Tag helicase. In the same report, Veaute and collaborators (*60*) analyzed by two-dimensional gel electrophoresis the structure of replication intermediates derived from circular duplex molecules containing either a [*c*,*s*]TT or a [6-4]TT. They concluded that photoproducts on the leading strand template, but not on the lagging strand template, blocked DNA replication forks. Their interpretation of the two-dimensional gel electrophoresis results was predicated on DNA replication proceeding by the action of a single fork in the majority of their SV40 origin-containing circular duplex molecules. If this premise were correct and the photoproduct on the leading strand template blocked further displacement of the replication fork, one would expect similar accumulations of gapped intermediates (Form II resistant to *Pst*I digestion) among replication products of both [6-4]TT-oriL and [6-4]TT-oriR. This prediction is not supported by the results depicted in Figure 6 for either the HeLa or the XP-V extracts. Polη-deficient extracts from XP-V cells are defective in bypass replication of [*c*,*s*]TT (*14*, *22*−*25*), but they generated excess amounts of radiolabeled Form II only during replication of the oriR substrates containing either the [*c*,*s*]TT or the [6-4]TT.

The diagrams illustrated in Figure 7 offer plausible explanations for the structure of intermediates of replication of circular duplex molecules carrying a site-specific photoproduct. These models incorporate findings from this and previous studies (*14*, *22*, *36*, *59*, *61*). They account for observations that DNA lesions interrupt, at least transiently, the synthesis of both the leading and the lagging strands. Blockage of the leading strand by the photoproduct (Figure 7A) does not stop progression of the replication fork. The uncoupling of leading and lagging strand synthesis creates an extended single-stranded DNA region on the 5′ side of the photoproduct. If and when bypass replication occurs, both

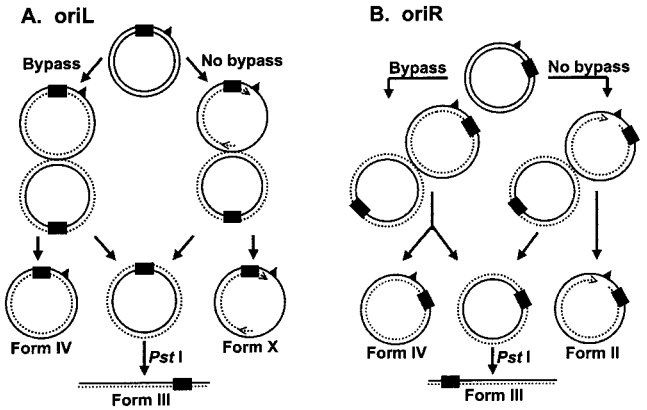

FIGURE 7: Models depicting the effects on replication products of a photoproduct on the template to the leading strand (oriL) or the lagging strand (oriR) of nascent DNA. The SV40 origin of replication in the closed circular molecules is represented by a filled rectangle, and the position of the photoproduct is indicated by the filled triangle. Solid lines depict circular parental strands and dotted lines the nascent DNA synthesized in vitro. Arrowheads indicate the direction of DNA polymerization. See text for details.

strands are completely replicated. Once the two forks merge at approximately 180° from the origin, the segregation of the two daughter molecules yields a *Pst*I-sensitive Form IV and a *Pst*I-resistant Form IV (successful bypass), or a partially single-stranded circular DNA molecule (Form X in Figure 7A). Because of slight differences in the rate of progression, the two replication forks do not merge at a fixed point in the circle from one molecule to another. Therefore, the population of Form X molecules with single-stranded regions of variable lengths generates a smear rather than a well-defined band of gapped molecules (compare the distribution of replication intermediates from constructs containing the [6-4]TT in Figures 3 and 5). Once the daughter molecules are segregated, lagging-strand synthesis on the damaged strand is also aborted.

When the photoproduct is on the template to the lagging strand (oriR), it interrupts the completion of a single Okazaki fragment (Figures 1B and 7B), but the fork continues replicating both strands of DNA around the circular duplex until it encounters the second fork. The segregation of the two daughter molecules yields the product of the undamaged strand (Form IV sensitive to *Pst*I digestion) and a bypass product (*Pst*I-resistant Form IV), or a gapped circular molecule (Form II). Close scrutiny of the gel fractionation of replication intermediates from oriL molecules (Figure 3), compared to those from oriR (Figure 5), indicated that accumulation of Form II molecules occurred primarily during the replication of oriR constructs. This finding was further substantiated by results gathered with XP-V extracts (Figure 6).

Form IV molecules resistant to *Pst*I were indicative of bypass replication. They were primarily observed when the photoproduct was a [*c*,*s*]TT and the extracts contained polη (Figures 3 and 5). Whether in the oriR constructs TLS occurred while the lagging strand template was still within replication fork-associated replication complexes, or through a gap-filling reaction after the dissociation of the daughter molecules cannot be discerned from the data presented here. For TLS to take place once leading strand synthesis is blocked by the [*c*,*s*]TT, the 3′ terminus of the nascent DNA strand must switch from the active site of the main polymerase to that of polη. It is not known whether this switch represents a kinetic step within the stalled replication complex, or requires the complete disassembly of the DNA synthesis machinery before polη can catalyze the bypass reaction. Despite the apparent complexity of these steps, it is possible that TLS is more likely to occur at a functional replication fork than once the fork is displaced away from the lesion, at least during replication of double-stranded circular duplexes in vitro.

Products of in vitro replication by a HeLa extract of molecules carrying a [6-4]TT on the template to the lagging strand were shown to be devoid of mutations (*44*). In light of the results presented here, the failure to detect targeted mutations at [6-4]TT is best explained by the reduced capacity of hypotonic extracts from human cells to bypass this photoproduct. The replication substrate used by others (*44*) did not allow distinction of bypass products from Form IV derived from the undamaged template strand. In this study, Form IV products were generated from [6-4]TT-oriL (Figure 3) and from [6-4]TT-oriR (Figure 5), but most of these molecules were sensitive to digestion by *Pst*I (Figure 4), demonstrating that they were generated primarily from the replication of the undamaged strand. Similar results were obtained with extracts from NER- (XP-A), MMR- (HCT116), and polη- deficient cells. All of the extracts used in this study (except those from XP-V cells) were competent to replicate the [*c*,*s*]TT dimer (*22*, *61*) on either template strand, but deficient in bypass replication of the [6-4]TT (results not shown). Even if the low NER and MMR activities present in HeLa extracts (*61*) removed the photoproduct from a fraction of the substrates prior to DNA replication in vitro, the daughter molecules generated from repaired substrates were sensitive to *Pst*I digestion (Figures 3 and 5).

In summary, the use of improved test substrates in this study afforded the demonstration that hypotonic extracts that are capable of efficient bypass of the [*c*,*s*]TT do not bypass the [6-4]TT very well. These findings might be related to sufficient leakage of polη from the nucleus during extract preparation to support bypass replication of the [*c*,*s*]TT. It is possible that polζ, or any other bypass polymerase that might replicate the [6-4]TT in vivo, is present in very low abundance and/or cannot be extracted well from the nucleus under standard methods of preparation of cell-free extracts. Information on the expression of polζ at the protein level, on potential posttranslation modifications following DNA damage, or on its cellular compartmentalization is not yet available. If translesion synthesis of the [6-4]TT occurs during in vitro replication of circular duplex molecules, it is very inefficient. The bypass efficiency for the [6-4]TT, estimated at 8−10% (Table 1), is likely to represent the upper limit of the range detectable in different extracts by the present technology.

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