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Discrimination between Translesion Synthesis and Template Switching during Bypass Replication of Thymine Dimers in Duplex DNA*

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The goal of this study was to determine whether bypass replication occurs by translesion synthesis or template switching (copy choice) when a duplex molecule carrying a single *cis,syn*-cyclobutane thymine dimer is replicated *in vitro* by human cell extracts. Circular heteroduplex DNA molecules were constructed to contain the SV40 origin of replication and a mismatch opposite to or nearby the dimer. Control molecules with only the mismatch were also prepared. Heteroduplexes were methylated at CpG islands and replicated *in vitro* (30 min). Following bisulfite treatment, the nascent DNA complementary to the dimer-containing template was distinguished from the other three strands by methylation-specific polymerase chain reaction. Cloning and sequencing of polymerase chain reaction products revealed that 80–98% carried the sequence predicted for translesion synthesis, with two adenines incorporated opposite the dimer. The fraction of clones with sequence predictive of template switching was reduced when extracts deficient in mismatch repair or nucleotide excision repair activities were used to replicate the heteroduplex molecules. These results support the conclusion that lesion bypass during *in vitro* replication of duplex DNA containing thymine dimers occurs by translesion synthesis.

Genomes are constantly damaged through thermal, chemical, and radiation-induced reactions that cause modifications in DNA structure. These can lead to mutations during replication of the damaged DNA. A network of DNA repair pathways and checkpoint responses minimize this risk. Both prokaryotes and eukaryotes are also endowed with DNA damage tolerance pathways that increase survival by facilitating the duplication of the genome, even in the presence of unrepaired lesions. These pathways include lesion bypass during semi-conservative DNA synthesis by mechanisms that are not yet completely understood (1, 2).

In the past 3 years, novel DNA polymerases have been identified and characterized in bacteria, yeast, and humans. These new enzymes have in common the property of extending primers beyond a template DNA lesion during replication *in vitro*. In *Escherichia coli*, they include the product of the *dinB* gene, namely DNA polymerase (pol)[1] IV (3), and UmuD′$_2$C (pol V) (4). *Saccharomyces cerevisiae* contains two or three specialized DNA polymerases encoded by the *REV3*, *REV7*, *REV1,* and *RAD30* genes. Rev3p and Rev7p are subunits of pol ζ (5). This DNA polymerase together with Rev1p, a primer-template-dependent deoxycytidyltransferase (6), appear to be responsible for most of the mutagenic events in *S. cerevisiae* (7). By contrast, the product of the *RAD30* gene (pol η) was shown to bypass cyclobutane thymine dimers efficiently by inserting adenines opposite this photoproduct (8).

The recent cloning of the human homologs of these new DNA polymerases has drawn the suggestion that the process of bypass replication of DNA lesions share common mechanisms in prokaryotes and eukaryotes (9, 10). Accordingly, Gibbs and collaborators (11, 12) have shown that the expression of *hREV3* or *hREV1* antisense RNAs in human fibroblasts reduces UV-induced mutagenesis. Loss of pol η (*hRad30A*) leads to the xeroderma pigmentosum variant (XP-V) syndrome (13–15). XP-V patients are prone to skin cancer in areas exposed to the sun (16), and their fibroblasts in culture are hypermutable by UV light (17–21). This indicates that pol η *in vivo* protects human cells from UV-induced mutagenesis by supporting the bypass of UV-induced lesions in much the same way as Rad30p does in yeast (8, 22). Also cloned recently were the human homolog of *E. coli* dinB (23), encoding pol θ (24), and another homolog of *S. cerevisiae RAD30*, termed *hRAD30B* (25). The latter is distinct from pol η (*hRAD30A*) but appears to encode also a bypass DNA polymerase, pol ι (26). Whether these novel DNA polymerases contribute to the bypass of different DNA lesions is still under investigation.

The preferred model of lesion bypass during DNA replication calls for a step of translesion synthesis catalyzed by one of the novel DNA polymerases described above (9, 10). One characteristic of these new enzymes is that they synthesize DNA with low processivity (27) and low fidelity (27, 29). This suggests that the bypass polymerases might replace the replicative polymerase blocked at the damage site, catalyze the addition of a few nucleotides across the lesion (their active sites are thought to be more tolerant of DNA distortions), and then dissociate from the primer-template (27, 28). At this point, the main replicase would return to the DNA growing point for efficient (processive and faithful) duplication of the undamaged DNA (9, 10, 27, 28). An alternative model of lesion bypass calls for the melting from the damaged template of the 3′ end of the blocked

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[1] The abbreviations used are: pol, polymerase; MMR, mismatch repair; NER, nucleotide excision repair; PCR, polymerase chain reaction; T^T, thymine dimer; UV, ultraviolet light; XP-V, xeroderma pigmentosum variant; bp, base pair; MET, methylated; UNMET, unmethylated.

strand and re-annealing to the complementary nascent DNA strand (copy choice). This template-switching model was first proposed by Higgins *et al*. (30) and Fujiwara and Tatsumi (31) to explain bypass replication of UV-induced lesions in irradiated human cells. Such a damage-avoidance mechanism has been proposed to operate in bacteria (32) and yeast (33). There is strong evidence that bypass polymerases, such as pol ζ (5) and pol η (8, 13, 14, 28, 34), carry out translesion synthesis in primer-extension assays *in vitro*. However, the possibility that template switching might occur during the replication of a duplex DNA, when the synthesis of both leading and lagging strands takes place, has not been formally excluded.

The template-switching model did not receive much attention until recent years because it was assumed that interruption of leading strand synthesis by a template lesion would result in complete blockage of the replication fork (35, 36). Studies using *in vitro* replication of duplex DNA, however, has provided evidence that uncoupling of leading and lagging strand synthesis occurs when leading strand synthesis is blocked by a template lesion (37–40). Under these conditions, the replication fork continues to move beyond the damaged site, extending the lagging strand by 1 to 2 thousand nucleotides beyond the lesion (40, 41). These findings, as well as evidence that extended single-stranded DNA regions are also formed during DNA replication in UV-irradiated mammalian cells (42, 43), suggested that a template-switching mechanism was indeed quite plausible. The appeal of such a lesion bypass pathway was its potential for high fidelity and for being independent of the nature of the blocking lesion.

In this study we designed and carried out experiments to determine whether translesion synthesis or template-strand switching is the primary mechanism by which a site-specific thymine dimer is bypassed during *in vitro* replication of a duplex DNA. Heteroduplex circular molecules with or without a single *cis,syn*-thymine dimer [T^T] on the template to the leading strand and a mismatch on the complementary template were fully methylated at CpG islands and replicated *in vitro* by several bypass-proficient human cell extracts. Leading strand was successfully distinguished from the lagging strand and methylated template strands by methylation-specific PCR, after the DNA was treated with bisulfite. Sequencing of cloned PCR products revealed that translesion synthesis, and not template switching, was the major mechanism by which *cis, syn*-thymine dimers were bypassed during *in vitro* replication.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Preparation of Cell-free Extracts—HeLa S3 cells were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Facility (University of North Carolina, Chapel Hill) or from the National Cell Culture Center (Minneapolis, MN). The IDH4 cell line was a gift from Dr. Jerry Shay (University of Texas Southwestern Medical Center). This cell line was generated by transformation with SV40 large T antigen of fibroblasts from an apparently normal human fetus (44). SV40-transformed XPA cells (XP12BE) were obtained from the NIGMS Human Genetic Mutant Cell Repository (GM4429). The human colorectal cancer cell line HCT116 (hMLH1 mutant, MMR defective; see Refs. 45 and 46) was obtained from Dr. Thomas A. Kunkel from the NIEHS, National Institutes of Health. Cells were grown in monolayer cultures at 37 °C in an atmosphere of 5% CO_2 in air. HCT116 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Life Technologies, Inc.) with 10% fetal bovine serum (HyClone Laboratories). The other cell lines were grown in Eagle's minimal essential medium, supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Growth medium for IDH4 cells also contained 1 μM dexamethasone (Sigma). Replication-competent cell extracts for DNA replication *in vitro* were prepared as published (47, 48).

Construction of Circular Heteroduplexes Molecules Containing a Mismatch and a Single T^T—The base changes required for the formation of the mismatch in the final heteroduplex (Fig. 1) were introduced in the sequence of the + strand of M13LeaSV (40). Mutated + strands for the construction of Lea-C and T^T-C were prepared essentially as described (40), except that the 20-mer inserted into M13mp2SV (49) by site-directed mutagenesis (50) was changed by a single base (5′-GAGCTCA**C**TTAGTCAGCTGC-3′). The mutated + strand in Lea-munI and T^T-munI were produced with the QuickChange™ site-directed mutagenesis kit (Stratagene). The 35-bp oligonucleotides 5′-CCGGCTCGAGCTCAATT**G**GTCAGCTGCGTATGTTG-3′ and 5′-CAACATACGCAGCTGAC**C**AATTGAGCTCGAGCCGG-3′ containing a point mutation at position 389 (bold and underlined) were used to introduce a unique *Mun*I site into M13LeaSV. Mutated products were verified by sequencing of the + strands. Mutated closed circular single-stranded DNA was purified and annealed with the oligonucleotide 3′-CTCGAG**T^T**AATCAGTCGACG-5′ previously phosphorylated at the 5′ end using T4 polynucleotide kinase (40). Then, second-strand synthesis, ligation, and purification in CsCl gradients were carried out according to published procedure (51). Control heteroduplex molecules (Lea-munI and Lea-C) were prepared in the same manner, using an oligonucleotide of identical sequence, but without the thymine dimer.

In Vitro DNA Replication—Heteroduplex DNA molecules were incubated with the bacterial *Sss*I methylase (New England Biolabs) for 2–3 h at 37 °C, under conditions suggested by the enzyme supplier for methylation of CpG sites. Complete methylation was confirmed by resistance of the closed circular heteroduplex molecules to digestion by *Aci*I (New England Biolabs), a restriction enzyme that cuts only unmethylated DNA.

In vitro replication of the methylated heteroduplexes was carried out in 25-μl reactions, as described previously (40), except that the concentration of dCTP was reduced to 50 μM. Reaction mixtures lacking SV40 large T antigen were used as negative controls of *in vitro* DNA replication. After incubation at 37 °C for 30 min, reactions were terminated by adding an equal volume of stop solution containing 2% SDS, 2 mg/ml proteinase K, and 50 mM EDTA. DNA was purified by using the QIAEX II Gel Extraction System (Qiagen). In experiments with T^T-munI and Lea-munI, the purified replication products were digested with *Mun*I (Roche Molecular Biochemicals) for 2–3 h at 37 °C. Aliquots of purified DNA samples (~10 ng) were fractionated in 1% agarose gels containing 0.2 μg/ml ethidium bromide. Dried gels were exposed to a phosphor screen that was later scanned by a PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA).

Bisulfite Treatment—Several protocols for bisulfite conversion of cytosine to uracil in single-stranded DNA have been described (52–56). In order to achieve efficient bisulfite conversion, the purified DNA was first digested with *Xmn*I restriction endonuclease (New England Biolabs) for 2–3 h at 37 °C. There are two *Xmn*I recognition sequences in the heteroduplex DNA used in these experiments (Fig. 1). The restricted DNA (18 μl, ~30 ng) was denatured by incubation in 0.3 M NaOH (addition of 2 μl of freshly prepared 3 M NaOH) for 15 min at 37 °C. Then, 200 μl of freshly prepared 5 M bisulfite solution, pH 5.0 (a mixture of 2.5 M metabisulfite and 500 mM hydroquinone; Sigma), was added to each reaction tube, followed by an incubation of 4 h at 50 °C. The bisulfite-treated DNA was desalted by using the Wizard® DNA clean-up system (Promega) and desulfonated by addition of 3 M NaOH to a final concentration of 0.3 M. After 15 min at 37 °C, the solution was neutralized by addition of 7.5 M NH_4Oac, pH 7.0, to a final concentration of 3 M. DNA was precipitated with ethanol and resuspended in 12 μl of 10 mM Tris, containing 1 mM EDTA, pH 8.0, and used immediately or stored at −20 °C.

PCR Amplification of the Bisulfite-treated DNA—The list of primers used in this study is shown in Table I. Primers were designed for both 5′→3′ and 3′→5′ strands. Strand-specific primers for the selective amplification of the replicated DNA (UNMET) or the methylated template (MET), nonselective primers, and the sequencing primer were designed using the GCG sequence analysis software package of the Wisconsin Genetics Computer Group (version 7). Primers were synthesized at the Pathology Oligonucleotide Synthesis Facility (University of North Carolina, Chapel Hill).

PCR was carried out in a Touchdown™ Thermal Cycler with a hot-start step that improved specificity. Reaction mixtures (50 μl) containing 3 μl of bisulfite-treated DNA, 10 pmol of each PCR primer, 10 nM of each dNTP, and buffer supplied by Qiagen were placed in the thermal cycler and hot-started at 94 °C for 4 min. After addition of 2.5 units of *Taq* polymerase (Qiagen) the denaturation step was continued for 2 min, followed by the touchdown protocol (denaturation at 94 °C for 30 s, annealing at *X* °C for 30 s, extension at 72 °C for 30 s). The initial annealing temperature of 61 °C was decreased at the rate of 1 °C for every PCR cycle until the targeted temperature was reached (optimal annealing temperature for the primers used; see Table I). At the target

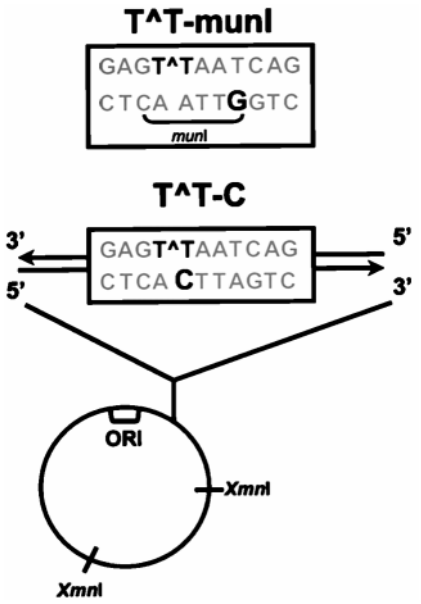

FIG. 1. **Schematic representation of the two constructs used as substrates for *in vitro* DNA replication.** M13leaSV, a 7.4-kilobase pair circular duplex molecule carrying the SV40 origin of replication (40), was modified to contain a single T^T in the − strand and a mismatched base on the + strand. The location of the T^T relative to the SV40 origin of replication placed the dimer on the template to the leading strand for the first replication fork to encounter the lesion. In T^T-C, a mismatched C was placed opposite the 5′-T of the dimer. In T^T-munI, a G:T mismatch was placed 3 bp downstream from the dimer to create a *Mun*I recognition site upon replication of the undamaged strand.

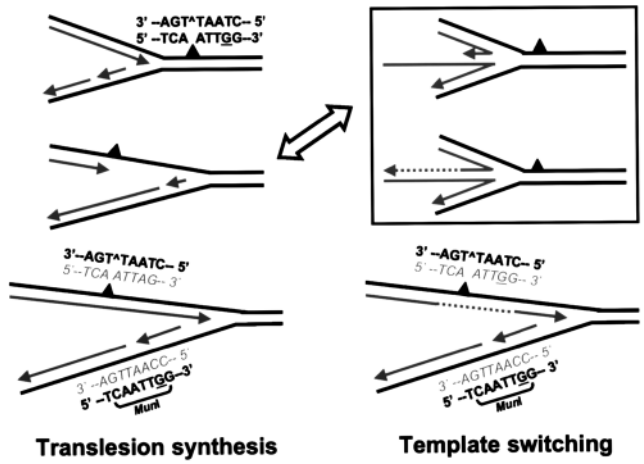

FIG. 2. **Predicted intermediates of bypass replication of a T^T blocking leading strand synthesis.** Uncoupling of lagging strand synthesis, displacement of the replication fork beyond the dimer, and formation of an extended single-stranded DNA region have been previously documented (41). This study sought to determine whether such structures could represent intermediates of bypass replication by template switching via the hypothetical configurations shown inside the *boxed inset.* The mismatched base on the DNA strand complementary to that carrying the T^T provided the sequence marker needed to determine whether extension of the leading strand beyond the dimer (bypass replication) occurred primarily by translesion synthesis or template switching. DNA sequences surrounding the dimer in the T^T-munI construct are shown in *bold* (template DNA) and in *italics* (daughter DNA) to illustrate the predicted sequence of the 5′→3′ leading strand following bypass replication according to one or the other of the two potential models. The completion of a *Mun*I recognition site upon replication of the 5′→3′ template strand containing the mismatched G should allow for the digestion of the resulting duplex prior to amplification and sequencing of the nascent leading strand.

temperature, 19–20 regular PCR cycles were performed, followed by a final extension step of 10 min at 72 °C. Negative controls for the strand-specific primers were performed with each PCR set. Aliquots (2 μl) of the PCRs were mixed with Ficoll loading buffer (3% Ficoll 400, 25 mM EDTA, 0.025% Orange G) and subjected to electrophoresis in 1.2% agarose gel containing 0.2 μg/ml ethidium bromide.

For direct sequencing, PCR products were purified using QIAquick™ PCR product purification system (Qiagen). PCR products obtained from 5′→3′ and 3′→5′ strands of template, and replicated DNA was directly sequenced using one of the PCR primers. PCR products obtained from the leading strand of replicated DNA were subcloned using TA Cloning Kit (Invitrogen). Plasmid DNA from individual clones were isolated using WIZARD PLUS Minipreps Kit (Promega), checked for the presence of an insertion, and sequenced using the sequencing primer (Table I). DNA sequencing was done at the University of North Carolina, Chapel Hill, Automated DNA Sequencing Facility on a model 377 DNA Sequencer (Perkin-Elmer) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase. The sequence data were analyzed using the GCG sequence analysis software package of the Wisconsin Genetics Computer Group (version 7).

RESULTS

Substrate Design—Two sets of circular heteroduplex DNA molecules were prepared for *in vitro* DNA replication (T^T-munI and T^T-C), each containing a control molecule lacking the T^T dimer (Lea-munI and Lea-C, respectively). In the T^T-munI and T^T-C substrates, the dimer was placed in the template to the leading strand (for the first replication fork encountering the lesion), 385 bp from the center of the SV40 origin of replication (Fig. 1). The two constructs differed only in the location of the mismatched bases. In T^T-munI, a T:G mismatch was at position 389, 3 bp away from the T^T dimer (3′-T^TAA**T**-5′:5′-AATT**G**-3′). This mismatch created a unique *Mun*I site in the replication product of the undamaged strand, but the replication product of the damaged strand remained resistant to *Mun*I. The second heteroduplex (T^T-C) contained a T:C mismatch opposite the T^T dimer (3′-T^**T**AA-5′:5′-A**C**TT-3′) at position 386. The single mismatch opposite (T^T-C) or nearby (T^T-munI) the T^T dimer was used as the marker of the undamaged strand to discriminate whether DNA replication past the dimer occurred by translesion synthesis or template switching. The diagram in Fig. 2 illustrates how these two mechanisms could be distinguished by sequencing the 5′→3′ leading strand of nascent DNA synthesized from T^T-munI. If translesion synthesis were the major mechanism by which the replication machinery bypassed the lesion, the expected sequence of the leading strand (complementary to the T^T-containing template) would be *AATTA* (*AATT* with the T^T-C construct). In case of template strand switching (replication across the lesion was avoided by using the undamaged lagging strand as template), the leading strand molecules would carry the information from the marked undamaged strand and display the sequence *AATTG* (*ACTT* with the T^T-C construct).

In Vitro Replication and Modification of DNA Strands—We chose to carry out the *in vitro* replication reactions for 30 min to maximize lesion bypass but to minimize the probability of a second round of replication (38, 40, 41, 57). Circular heteroduplexes were first treated *in vitro* with *Sss*I, an enzyme that methylates cytosines exclusively at CpG dinucleotides in double-stranded DNA (58). Resistance to digestion by *Aci*I confirmed that the heteroduplex DNA was efficiently methylated on both strands (there are 47 *Aci*I recognition sites, 5′-CCGC-3′, in the 7.4-kilobase pair circular molecule). Next, we determined that the methylated heteroduplexes could be replicated *in vitro* by human cell-free extracts. SV40 large T antigen-dependent replication of the fully methylated heteroduplexes represented 25–75% (varying with the extract and substrate used) of that obtained in parallel reactions containing unmethylated DNA (results not shown).

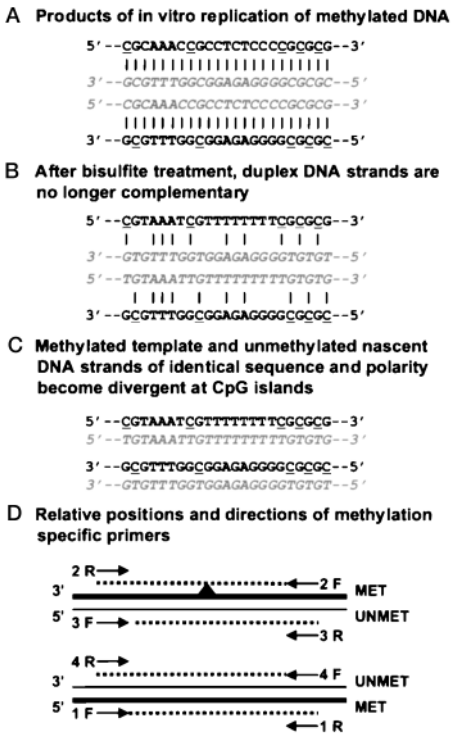

FIG. 3. **Basis for selective PCR amplification of each of four DNA strands of a replicated duplex molecule.** *A, Sss*I methylation of template DNA at CpG islands (**C**) does not interfere with semiconservative DNA replication. *B,* bisulfite treatment leads to deamination of unmethylated cytosines to uracil (same base pairing characteristics as thymine). This results in loss of base pairing between the two initially complementary strands. *C,* sequence divergence at CpG islands between template and nascent DNA of identical polarity allows for the design of PCR primers that amplify specifically one strand over the other. Methylated cytosines in template DNA (*bold*) are *underlined,* and the nascent DNA is shown in *italics. D,* diagram indicating the relative positions and directions of primers used during methylation-specific PCR to amplify individual strands of DNA replicated *in vitro*. *Thick lines* represent methylated template strands (*MET*); the *filled triangle* on the 3′→5′ template strand represents the site-specific thymine dimer. *Thin lines* stand for the DNA newly synthesized *in vitro*, thereby unmethylated (*UNMET*). Forward (*F*) and reverse (*R*) primers are numbered according to their presentation in Table I. *Dotted lines* represent DNA synthesized during PCR.

Genomic sequencing after bisulfite modification was developed by Frommer and collaborators (52, 53). The method is based on sodium bisulfite-mediated conversion of unmethylated cytosine to uracil under conditions whereby 5-methylcytosines are resistant to this conversion. We used this approach to distinguish the products of DNA replication *in vitro* (3′→5′ and 5′→3′ newly synthesized strands) from each other and from their template strands. Single round of replication of double-stranded templates fully methylated at CpG islands yields two product molecules each containing one methylated parental strand and one unmethylated newly synthesized strand (Fig. 3*A*). Bisulfite reaction converts all unmethylated cytosines to uracils, whether they are in the newly synthesized or the template strands. There are two advantages of methylation and bisulfite modification of *in vitro* replicated DNA. After treatment, the duplex DNA strands are no longer complementary (Fig. 3*B*), and methylated template and unmethylated nascent DNA strands of identical sequence and polarity become divergent at CpG islands (Fig. 3*C*). Thus, bisulfite-treated products of replication of duplex DNA methylated at CpG islands become a mixture of four different strands that can be selectively amplified by PCR with strand-specific primers.

Primer Design—PCR primers were designed as described by Herman *et al.* (59). They are listed in Table I, and their relative hybridization positions on the target DNA strands are shown in Fig. 3*D*. These primers were able to amplify efficiently both the bisulfite-treated methylated and unmethylated strands of M13leaSV (the parent plasmid used to generate heteroduplexes). A nonselective primer pair was also designed to check the methylation status of the heteroduplex template strands after the incubation with human cell extracts. This was necessary to rule out possible contamination of leading strand DNA with template strands demethylated during the *in vitro* replication reaction. In control experiments, methylated T^T-munI DNA was incubated for 30 min with either HeLa or IDH4 extracts in the absence of SV40 large T antigen (no semiconservative DNA replication). After bisulfite treatment, the 5′→3′ strands were PCR-amplified using nonselective primers, and the PCR products were purified and sequenced directly. Results (not shown) revealed that methylated molecules retained the CpG islands after incubation with human cell extracts. Sequencing histograms showed that each C peak at CpG islands contained only one signal. No evidence was found for a secondary (shadow) peak representing demethylated cytosines (*i.e.* uracils) at positions corresponding to CpG islands. These results clearly indicated that under conditions used for *in vitro* replication the template DNA molecules remained methylated and should not interfere with results obtained during the sequencing of the unmethylated newly synthesized DNA.

Reconstruction experiments were used to estimate the specificity of primers to amplify only the targeted strand among the four expected to be present in the *in vitro* replication mixture (Fig. 4). Use of touchdown PCR with a hot start improved the specificity of primers, and no PCR products were detected in the reactions containing negative controls. Unmethylated M13leaSV was premixed with 10× the mass of methylated M13leaSV. This mixture was treated with bisulfite, and aliquots were subjected to PCR using the 5′–3′-UNMET primer set (Fig. 4*A*). Separate PCRs containing either bisulfite-treated unmethylated DNA or 10× the mass of bisulfite-treated methylated DNA were used as controls. Note that only the bisulfite-treated, unmethylated DNA was amplified with the 5′–3′-UNMET primer set. Fig. 4*B* illustrates the selective amplification of the bisulfite-treated methylated DNA with the 5′–3′-MET primer set. In this case, the methylated DNA that was treated with bisulfite was mixed with 10× the mass of the untreated methylated DNA. Only the bisulfite-treated DNA was amplified with the 5′–3′-MET primer set.

Next, we examined the specificity of the 5′–3′-UNMET primers to amplify bisulfite-treated DNA from an *in vitro* replication mixture containing the IDH4 extract and methylated Lea-C as the substrate (Fig. 4*C*). DNA from *in vitro* replication reactions in which methylated Lea-C was incubated with the same extract in the absence of SV40 large T antigen (no replication) was used as an additional negative control. Efficient PCR amplification with the 5′–3′-UNMET primers was only observed with DNA from *in vitro* replication reactions when the addition of SV40 large T antigen promoted the replication of the methylated Lea-C substrate. Direct sequencing of PCR products obtained with 5′–3′-UNMET and 3′–5′-UNMET primer pairs revealed that all CpG dinucleotides were converted into UpGs during bisulfite treatment and then PCR-amplified as TpGs. Direct sequencing also revealed that the 5′→3′ leading strand synthesized from the region of interest (carrying the mismatch 3′-T**T**AA-5′:5′-A**C**TT-3′) was 5′-*AATT*-3′, whereas the 3′→5′ lagging strand was 3′-*TGAA*-5′ (underlining added for emphasis). Both template strands were amplified from the same *in vitro* replication reaction using 5′–3′-

TABLE I

Primer sets for methylation-specific PCR amplification of DNA strands from replicated duplex DNA (see Figs. 1 and 3D)

Underlined nucleotides represent positions protected from deamination by *Sss*I methylation of template DNA but modified by bisulfite treatment in replicated DNA; sequence differences between primers and DNA not treated with bisulfite are in boldface. See Fig. 3*D* for schematic representation of the relative positions and directions of these primers.

Primers	Amplified DNA strand	Forward primers 5′→3′	Reverse primers 5′→3′	Product length	Optimal annealing temperature
					°C
1) 5′–3′-MET	Template	CGTAAATCGTTTTTTTTC	ACACCGCTTCTAATACC	436	50.4
2) 3′–5′-MET	Template	ATGGGCGTATCGTAATC	GAACTAAAAACGAACAATAAAC	461	51.7
3) 5′–3′-UNMET	Leading	TATGGGGTGGAGAATGGGT	CACAACTATTAAAAAAAACAATCAATACA	570	52.0
4) 3′–5′-UNMET	Lagging	GTGTGGGTTTTTTTGTTATTATGT	CTAACACCCAATACACAAACCA	375	51.5
5) 5′–3′ Non-selective	Template + Leading	TAGGCGGTGAAGGGTAA	AATACCGAAAACCAAACAA	495	51.6
6) Sequencing	Leading	CCAACTAACAAAAAAAAATATACTACAAAACA			

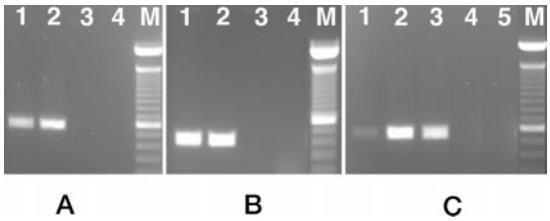

FIG. 4. **Specificity of PCR primers (see Table I).** *A,* selective amplification of bisulfite-treated, unmethylated DNA by the 5′-3′-UNMET primer set. *Lane 1,* mixture of 4 ng of bisulfite-treated, unmethylated M13leaSV DNA with 40 ng of bisulfite-treated methylated M13leaSV; *lane 2,* 4 ng of bisulfite-treated, unmethylated M13leaSV DNA; *lane 3,* 40 ng of bisulfate-treated, methylated M13leaSV; *lane 4,* PCR reaction with no DNA. *B,* selective amplification of bisulfite-treated, methylated DNA by the 5′–3′-MET primer set. *Lane 1,* mixture of 4 ng of bisulfite-treated, and 40 ng of untreated, methylated M13leaSV DNA; *lane 2,* 4 ng of bisulfite-treated, methylated M13leaSV DNA; *lane 3,* 40 ng of methylated M13leaSV not treated with bisulfite; *lane 4,* no DNA. *C,* selective amplification of unmethylated nascent DNA with the 5′–3′-UNMET primer set after replication *in vitro* of methylated Lea-C DNA. *Lane 1,* bisulfite-treated DNA from *in vitro* replication reactions containing methylated Lea-C DNA but lacking SV40 large T antigen; *lanes 2* and *3*, bisulfite-treated DNA after SV40 large T antigen-dependent *in vitro* replication of methylated Lea-C DNA; *lane 4,* bisulfite-treated methylated Lea-C DNA not incubated *in vitro*; *lane 5,* no DNA. *M* marks the lanes containing size markers (100-bp DNA ladder, Life Technologies, Inc.).

MET and 3′–5′-MET primer sets and sequenced directly. Results showed that all CpG dinucleotides were protected by methylation in both template strands during bisulfite reaction. The 5′-A**C**TT-3′ template strand (mismatch-containing region) was amplified during PCR as 5′-A**T**TT-3′ (unmethylated cytosine was converted to U and then to T) and 3′-T**T**AA-5′ template strand was amplified as 3′-T**T**AA-5′.

Sequence Analysis of in Vitro Replication Products of Undamaged Heteroduplexes—Methylated Lea-munI (3′-TTAA**T**-5′:5′-AATT**G**-3′) was replicated *in vitro* by HeLa and IDH4 extracts and treated with bisulfite after *Xmn*I and *Mun*I digestion. As emphasized above, the mismatch in this construction leads to the introduction of a recognition site for *Mun*I in the replication product of the 5′→3′ template strand. After complete digestion with *Mun*I, neither the 3′→5′ lagging nascent strand nor the 5′→3′ template strand in replicated molecules should be available for PCR amplification. After bisulfite treatment, the 5′→3′ leading nascent strand from three independent *in vitro* replication reactions with methylated Lea-munI was amplified with the 5′–3′-UNMET primers and directly sequenced. This analysis confirmed that the targeted replicated strands were amplified because ~90–100% of the CpG dinucleotides in the amplified DNA was converted to TpGs. For sequencing analysis of individual molecules, PCR products were obtained from four independent *in vitro* replication reactions conducted with one of two human cell extracts (IDH4 and HeLa) and cloned. Approximately 20–25 cloned molecules from each reaction were sequenced, and the results are shown in Table II. The data revealed that 91 (HeLa, $n = 45$) and 98% (IDH4, $n = 46$) of sequenced molecules displayed the expected sequence *AATTA*. Similar results were obtained when the second heteroduplex, Lea-C (3′-T**T**AA-5′:5′-A**C**TT-3′), was replicated by the IDH4 extract. Among the 18 sequenced clones, 17 (94%) displayed the expected sequence *AATT* and only one (6%) showed the *ACTT* sequence (Table IV). We were surprised to find leading strand clones carrying the sequence *ACTT* (Lea-C; 6% in IDH4, Table IV) or the sequence *AATTG* (Lea-munI; 9% in HeLa and 2% in IDH4, Table II) among products of replication of the undamaged constructs. Therefore, we consider whether these clones could reflect the activity of mismatch repair (MMR) on the heteroduplex prior to DNA replication (60). The experiments were repeated with an extract of HCT116, an MMR-deficient cell line, and the results showed that all 49 sequenced clones (two independent *in vitro* replication reactions) carried the sequence *AATTA* (Table II).

Sequence Analysis of in Vitro Replication Products of T^T Dimer-containing Heteroduplexes—T^TmunI heteroduplex molecules were incubated *in vitro* with bypass-proficient extracts from HeLa and IDH4. Newly synthesized leading strand molecules were analyzed as described for the corresponding undamaged heteroduplex molecules. PCR products obtained from three independent *in vitro* replications were sequenced directly. Sequence histograms showed that the peak corresponding to the 3′- *A* at the 5′-*AATTA*-3′ region contained one strong A signal and a smaller G peak, indicating that the population of amplified molecules was heterogeneous in this region. After cloning of PCR products and sequencing of individual molecules, the data summarized in Table III were obtained. Among the molecules synthesized by HeLa ($n = 40$) and IDH4 ($n = 46$), 80 and 87% displayed the sequence *AATTA*, which was consistent with bypass replication taking place by translesion synthesis (Fig. 2). Clones carrying the sequence *AATTG* constituted 20% in HeLa and 13% in IDH4. According to our experimental design, the detection of *AATTG* sequences among the bypass products would indicate that direct damage bypass could be avoided by a template-switching mechanism. Before reaching the conclusion that such pathway was functioning during the replication of a small fraction of heteroduplex molecules, it was important to determine whether DNA repair activities could be distorting the experimental results. Both IDH4 and HeLa extracts are proficient in nucleotide excision repair (NER) and MMR. The heteroduplexes used in this study carry a compound lesion (a mismatch opposite the

TABLE II
Sequence of the 5′ → 3′ leading strand of nascent DNA from Lea-munI replicated in vitro by different extracts

		Lea-munI 3′-TTAAT-5′ 5′-AATTG-3′		
Extract	MMR capability[a]	No. of sequenced clones[b]	No. (%) of clones with sequence:	
			AATTA	**AATTG**
HeLa	Proficient	45	41 (91%)	4 (9%)
IDH4	Proficient	46	45 (98%)	1 (2%)
HCT116	Deficient	49	49 (100%)	0 (0%)

[a] MMR capability of these extract was confirmed by Dr. Alan Clark in Dr. Thomas A. Kunkel's laboratory (NIEHS, National Institutes of Health).
[b] Clones recovered from two independent experiments.

TABLE III
Sequence of the 5′→3′ leading strand of nascent DNA from T^T-munI replicated in vitro by different extracts

		T^TmunI 3′-T^TAAT-5′ 5′-A ATTG-3′		
Extract	Repair capability[a]	No. of sequenced clones[b]	No. (%) of clones with sequence:	
			AATTA (translesion synthesis)	**AATTG** (template switching?)
HeLa	MMR and NER-proficient	40	32 (80%)	8 (20%)
IDH4	MMR and NER-proficient	46	40 (87%)	6 (13%)
HCT116	MMR-deficient	46	43 (93%)	3 (7%)
XPA	NER-deficient	45	44 (98%)	1 (2%)

[a] Indicates proficiency or deficiency in MMR and NER.
[b] Clones recovered from two independent experiments.

T^T dimer), and such lesions are better substrates for NER than the simple T^T (61). If some molecules of T^T-munI (3′-T^TAA**T**-5′:5′-AATT**G**-3′) were repaired by NER to 3′-TTAA**C**-5′:5′-AATT**G**-3′, the replication of the repaired 3′-TTAA**C**-5′ strand would generate leading strand molecules with the sequence *AATTG*. If the repaired and replicated molecules escaped *Mun*I digestion, then AATTG clones could be recovered in our experiments without template switching having occurred during bypass replication. We also considered that if *AATTG* clones resulted from bypass replication by template switching, but post-replication repair corrected the mismatch by copying the template strand (3′-T^TAA**T**-5′:5′-*AATTG*-3′ repaired to 3′-T^TAA**T**-5′:5′-*AATTA*-3′), then our results could be distorted in favor of translesion synthesis. If that was the case, the percentage of *AATTG* clones should increase when MMR-deficient extracts were used to replicate the heteroduplex molecules. Therefore, these experiments were repeated with extracts from NER-deficient (XPA) and MMR-deficient (HCT116) cells. The data in Table III show that 93% of the leading strand molecules displayed the sequence *AATTA* and only 7% *AATTG* when *in vitro* replication was supported by the HCT116 extract. Therefore, a reduction and not an increase in the number of *AATTG* clones was observed in the absence of MMR activity. This observation strengthened the suspicion that prereplicative NER (and not template switching) could be the major source of the observed AATTG clones. This was confirmed by the results with an extract from XPA cells (NER-deficient). Among the 45 clones sequenced, only one (2%) displayed the sequence *AATTG* (Table III).

The results presented in Tables II and III strongly suggest that bypass replication of a single *cis,syn*-thymine dimer in duplex DNA occurs primarily by translesion synthesis. This conclusion was confirmed by the results obtained with the T^T-C construct (3′-T^**T**AA-5′:5′-A**C**TT-3′) and shown in Table IV. After *in vitro* replication by IDH4 and HCT116 extracts, the sequence of the leading strand was found to be *AATT* in 84% (IDH4, $n = 56$) and 82% (HCT116, $n = 39$) of the clones.

DISCUSSION

The significance of the data reported here rests on the demonstration that human replication complexes carry out bypass replication of a pyrimidine dimer in duplex DNA by translesion synthesis. Although this conclusion may seem obvious to many, this is the first analytical study designed to test this assertion experimentally. Recent discoveries of several novel DNA polymerases with bypass replication capability in primer extension reactions reinforced the expectation that polymerization across non-instructive lesions in duplex DNA is the norm. In addition, the *in vitro* replication of single-stranded vectors containing a single acetylaminofluorene adduct demonstrated that translesion synthesis through and beyond the damaged site can be catalyzed by extracts from bypass-proficient human cells (34). However, other pieces of evidence kept pointing to an alternative mechanism, mainly template switching, as a plausible pathway of damage tolerance when lesions are encountered during the replication of duplex DNA.

The potential co-existence in the same replication fork of a newly synthesized daughter DNA, with the same sequence and polarity as the lesion-containing template, was thought to hold the key to the mechanism by which DNA polymerization could be guided beyond the blocking lesion (Fig. 2). When this model was initially proposed (30, 31), however, it was met with skepticism. One of the experimental evidence presented in support of the model was the detection by electron microscopy of four-armed replication forks (see *boxed inset* in Fig. 2). These structures, however, could have been formed in solution by DNA branch migration instead of representing true intermediates of bypass replication. In the absence of conclusive proof in favor of or against template switching, this model remained appealing because it envisioned an error-free alternative for the completion of DNA replication, despite the presence of DNA damage in the genome. *In vitro* replication studies aimed at detecting replication past site-specific DNA lesions demonstrated that blockage of the leading strand at a damaged site did not interrupt the progression of the replication fork (37–40). In a large fraction of the replicating molecules, the attendant DNA synthesis resulted in polymerization of the lagging strand beyond the lesion, even before bypass could take place (41). These observations made clear that the necessary prerequisite for template switching, *i.e.* an undamaged 3′→5′ nascent strand to serve as the alternative template to the growing leading

TABLE IV
Sequence of the 5′→3′ leading strand of nascent DNA from Lea-C and T^T-C replicated in vitro

Extract	Lea-C 3′-TTAA-5′ 5′-AC̲TT-3′			T^T-C 3′-T^TAA-5′ 5′-A C̲TT-3′		
	No. of sequenced clones	No. (%) of clones with sequence		No. of sequenced clones[a]	No. (%) of clones with sequence	
		AATT	**AC̲TT**		**AATT** (translesion synthesis)	**AC̲TT** (template switching?)
IDH4	18	17 (94%)	1 (6%)	56	47 (84%)	9 (16%)
HCT116				39	32 (82%)	7 (18%)

[a] Clones recovered from two independent experiments.

strand, was indeed present at the damaged site. Therefore, it became important to design experiments to detect whether bypass replication by template switching was occurring *in vitro*.

The first experimental challenge was to mark the template strands, so that we could determine which one directed the synthesis of the DNA later found opposite the T^T. This was the purpose of adding a mismatched base opposite to or nearby the dimer in the two constructs used in this study (Figs. 1 and 2). Once bypass had taken place, we needed to determine without ambiguity the sequence of the newly synthesized leading strand. By using technology developed to study DNA methylation patterns, it was possible to distinguish the four strands of the duplex DNA and to amplify them selectively. The principles of this approach are illustrated in Fig. 3. Methylation protection of bisulfite deamination of cytosines, in stretches of the template strands containing at least three CpG islands, provided sufficient sequence divergence from the newly synthesized DNA, which prior to chemical treatment had an identical sequence and polarity. The positive and negative controls illustrated in Fig. 4, and subsequent sequencing analyses, demonstrated that the strand-specific primers listed in Table I indeed amplified only the targeted strand. These results set the stage for the determination of the sequence of interest, that is the one carried by the 5′→3′ leading strand complementary to the dimer-containing template. We cloned PCR products and sequenced individual molecules so that both major and minor events could be detected.

The final results shown in Tables II–IV strongly support the conclusion that translesion synthesis, most likely catalyzed by pol η, was responsible for the insertion of adenines opposite the T^T in >80% of the bypass products, without transfer of sequence information from the alternative template. Furthermore, the fraction of bypass products with the sequence predictive of template switching was dependent on the capability of the extract for DNA repair. The use of human cell extracts that were deficient in NER or MMR resulted in reductions in the number of clones carrying the sequence information of the nascent lagging strand. This finding suggested that the *AATTG* (*AC̲TT*) clones were not the product of template switching during bypass but instead were generated by DNA repair occurring prior to DNA replication. Remaining to be explained is why the repaired and replicated molecules carrying the sequence 3′-TTAA**C̲**-5′:5′-*AATT**G̲***-3′ were amplified by methylation-specific PCR when they should have been digested by *Mun*I. Although we optimized the reaction conditions for complete digestion of molecules carrying the *Mun*I recognition sequence, it is possible that some products of *in vitro* replication could have escaped digestion due to proteins remaining bound to the replicated DNA, even after its purification with the QIAEX II Gel Extraction System. The interpretation that template switching can occur in a small fraction of the replicating molecules, however, cannot be completely dismissed at this point. It can be argued that replication-competent extracts prepared from different human cell lines might differ in their capability to support template switching relative to translesion synthesis.

The maintenance of genetic stability requires that DNA replication be carried out with the highest degree of fidelity. This is accomplished by the combined action of DNA polymerases with low frequency of errors, proofreading activities, and post-replication repair. These functions depend on the recognition of correct base pairing between the template and the nascent strands. However, when the replication machinery encounters damaged sites in DNA, less stringent base pairing conditions must be accommodated (28, 29), if DNA synthesis across and beyond the lesion is to take place. This balance between high fidelity during the replication of normal DNA and the need to complete replication even in the presence of DNA lesions requires the concerted effort of different DNA polymerases. Even though the regulation of DNA polymerase switching at sites of blocked DNA replication is not yet understood, the results reported here demonstrate that translesion synthesis is the major mechanism by which a cyclobutane thymine dimer is bypassed during *in vitro* replication.

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