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Differential replication of a single, UV-induced lesion in the leading or lagging strand by a human cell extract: Fork uncoupling or gap formation

(skin cancer/mutation/DNA polymerase/simian virus 40)

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ABSTRACT We have constructed simian virus 40 minireplicons containing uniquely placed *cis,syn*-thymine dimers (T<>T) for the analysis of leading- and lagging-strand bypass replication. Assaying for replication in a human cell-free extract through the analysis of full-size labeled product molecules and restriction fragments spanning the T<>T site resulted in the following findings: (*i*) The primary site of synthesis blockage with T<>T in either the leading or lagging strand was one nucleotide before the lesion. (*ii*) Replicative bypass of T<>T was detected in both leading and lagging strands. The efficiency of synthesis past T<>T was 22% for leading-strand T<>T and 13% for lagging-strand T<>T. (*iii*) The lagging-strand T<>T resulted in blocked retrograde synthesis with the replication fork proceeding past the lesion, resulting in daughter molecules containing small gaps (form II′ DNA). (*iv*) With T<>T in the leading-strand template, both the leading and lagging strands were blocked, representing a stalled replication fork. Uncoupling of the concerted synthesis of the two strands of the replication fork was observed, resulting in preferential elongation of the undamaged lagging strand. These data support a model of selective reinitiation downstream from the lesion on lagging strands due to Okazaki synthesis, with no reinitiation close to the damage site on leading strands [Meneghini, R. & Hanawalt, P. C. (1976) *Biochim. Biophys. Acta* 425, 428–437].

Short wavelength UV irradiation (UVC) at 254 nm and the resulting DNA photochemistry have been correlated with mutagenesis, carcinogenesis, and cell death due to replication of unrepaired lesions in the DNA template (1). The mechanism of replication of damaged DNA in mammalian cells has been investigated through the study of UVC-irradiated cell cultures and by using UVC-damaged viral shuttle vectors such as simian virus 40 (SV40) and minute-virus-of-mice as probes (2). These experiments led to the proposal of different models. (*i*) Replication forks on damaged templates do not pause or stop at sites of UV damage (3, 4). (*ii*) Cyclobutane *cis,syn*-pyrimidine dimers (Pyr<>Pyr) always block replication forks, irrespective of their location (5, 6). (*iii*) Leading-strand Pyr<>Pyr block replication fork progression, while lagging-strand Pyr<>Pyr inhibit completion of Okazaki fragments with replication forks proceeding past the lesions (7, 8). Despite these investigations, a basic understanding of replication of damaged DNA in normal human cells is lacking (2).

In vitro studies using primed single-stranded DNA templates and purified DNA polymerases (9–13) have demonstrated the existence of both stalled replication intermediates and translesion synthesis. These studies involved DNA synthesis on primed templates rather than semiconservative coordinated replication of leading and lagging strands at a *bona fide* replication fork. *In vitro* replication of SV40-based minireplicons carrying randomly distributed, UVC-induced Pyr<>Pyr has been analyzed in human cell extracts (14, 15). A UV fluence-dependent inhibition of covalently closed circular DNA (form I DNA) synthesis was observed, which confirmed the blocking effect of such lesions on DNA replication. The presence of a UV endonuclease V from phage T4 (T4 UV endo)-sensitive product implied that replication of Pyr<>Pyr occurred in such a system. A detailed examination of the molecular mechanism of UV damage processing has become possible with the development of site-specific lesion-containing DNA (11–13). Thus, we now report sites of blockage and rates of bypass of individual lesions in the leading and lagging strands.

The most prevalent DNA modification resulting from UVC irradiation is the *cis,syn*-thymine dimer (T<>T), which has been a paradigm DNA lesion (16). We have prepared plasmids containing the SV40 origin of replication together with uniquely placed T<>T and have analyzed their *in vitro* replication in a human cell-free extract. We have developed these assays to study the mechanism of replication of DNA damaged in either the leading or lagging strands of a human replication fork. Specifically, we have determined the site of blockage of strand elongation on T<>T-containing DNA templates, compared the bypass of T<>T in the leading and lagging strands, and analyzed the potential uncoupling of coordinated leading- and lagging-strand synthesis at T<>T-blocked replication forks.

MATERIALS AND METHODS

Enzymes and Reagents. HeLa cytoplasmic extract was prepared as published (17). SV40 large tumor (T) antigen (TAg) was from Molecular Biology Resources (Milwaukee). Restriction endonucleases were from New England Biolabs. $[\alpha\text{-}^{32}\text{P}]$-dCTP (6000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Deoxyribonucleoside triphosphates were from Pharmacia. T4 DNA polymerase and T4 DNA ligase were from Boehringer Mannheim.

Double-Stranded Circular SV40 Minireplicons with Single, Site-Specific T<>T in Leading or Lagging Template Strands. The SV40 origin of replication (18) was inserted between the *Bam*HI and *Hin*dIII sites of both pBluescript II KS(−) and pBluescript II KS(+) yielding pKSori(−) and pKSori(+). A 20-nt sequence complementary to an oligonucleotide containing a single, site-specific T<>T was inserted 336 nt from the SV40 origin in pKSori(−) and 345 nt from the SV40 origin in pKSori(+), yielding pKSoriD(−) and pKSoriD(+), respec-

Abbreviations: T<>T, *cis,syn*-thymine dimer; Pyr<>Pyr, cyclobutane *cis,syn*-pyrimidine; UVC, short wavelength ultraviolet light; SV40, simian virus 40; T4 UV endo, UV endonuclease V from bacteriophage T4; UM, unmodified; form I, covalently closed circular DNA; form II, nicked circular DNA; TAg, SV40 large tumor (T) antigen.

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tively. The primer (pGCTCGAGCTCAAT<>TAGTCAG) was synthesized by the building-block method (19, 20).

The 20-nt T<>T-containing primer, and its unmodified (UM) TT-containing counterpart were separately annealed to circular single-stranded DNAs containing the complementary sequence downstream from the SV40 origin. The primers were extended with T4 DNA polymerase, and the resulting nicked circular DNA (form II) molecules were joined by T4 DNA ligase *in situ* (21). Double-stranded DNA was purified from products of incomplete synthesis (22). To prepare fully methylated template DNA, 1.25 μg of semisynthetic double-stranded DNA was treated with 0.05 unit of DNA adenine methylase (New England Biolabs) per ml and 0.375 mM *S*-adenosylmethionine (New England Biolabs) for 60 min at 37°C in 200 μl of the buffer provided by the supplier.

***In Vitro* Replication Assay.** Reactions were performed at 37°C in 30 mM Hepes containing 15 mM NaH_2PO_4/Na_2HPO_4; 7 mM $MgCl_2$ (pH 7.5); 100 μM each dATP, dCTP, dGTP, and dTTP; 20–50 μCi of [α-^{32}P]dCTP; 0.2 mM each of CTP, GTP, and UTP; 4 mM ATP; 40 mM creatine phosphate (Sigma); and 100 μg of creatine phosphokinase (Sigma), 0.08 mg of TAg, 1.6 μg of DNA template, and 5 mg of HeLa cytoplasmic extract per ml (17). All assays were performed in triplicate, and quantities are expressed ±SD.

To ensure that label incorporation was due to semiconservative DNA synthesis, a product mixture derived from fully methylated DNA template (see above) was treated with *Dpn* I restriction endonuclease, resulting in digestion of only a background quantity of labeled DNA (data not shown).

Determination of Replicative Bypass and Uncoupling Efficiencies. Dried gels were exposed to phosphor-imaging plates, which were scanned as 16-bit images with a Molecular Dynamics model 400 PhosphorImager with IMAGE QUANT software. Relative ratios of form I and form II DNA were measured in the presence and absence of T4 UV endo to determine the fraction of product form I molecules containing T<>T. Background due to nonreplicative incorporation of label, determined from reactions without TAg, was subtracted.

The conversion of form I molecules to form II by T4 UV endo was used to calculate the relative level of replication of templates containing T<>T as follows:

$$I_{T<>T} = 2 \times I_{T4sensitive}, \quad [1]$$

where $I_{T<>T}$ is the fully replicated T<>T-derived form I product, and $I_{T4sensitive}$ is the product form I DNA nicked by T4 UV endo.

The replicated T<>T-containing template equals 2 times the T4 UV endo-sensitive form I because only one of the two daughter molecules produced from a single parent molecule contains T<>T. The slope of a plot of $I_{T<>T}$ as a function of time gives the rate of replication of a T<>T-containing template.

The level of uncoupling (synthesis templated by the undamaged strand in the absence of completion of synthesis of the T<>T-containing strand) was derived from the product form I DNA lacking T<>T as follows:

$$U = I_{total} - I_{T<>T}, \quad [2]$$

where $I_{T<>T}$ is the fully replicated T<>T-derived form I product, I_{total} is the total form I product DNA, and U is the form I DNA from uncoupled synthesis of the undamaged strand.

This quantity is the undamaged form I DNA in excess of that accounted for by the complete replication of T<>T-containing parent molecules. The slope of a plot of U as a function of time gives the rate of accumulation of uncoupled replication product. When applied to synthesis using the lagging-strand T<>T template, Eq. 2 indirectly yields the rate of formation of gapped molecules (U = gapped molecules).

Relative rates are calculated from slopes of plots of the intensities of gel bands in the linear regime of time-course experiments as determined by linear least-squares regression analysis ($r^2 > 0.9$). These rates are based on pixel values (IMAGE QUANT software) and apply to relative comparisons within each experiment.

Sequencing Gel Analysis of Restriction Fragments. Labeled DNA samples obtained from *in vitro* synthesis reactions were treated with two different combinations of three restriction endonucleases: (*i*) *Ase* I, *Bsa* I, and *Xho* I; and (*ii*) *Afl* III, *Hae* II, and *Pvu* II. This analysis yields a high-resolution map of the

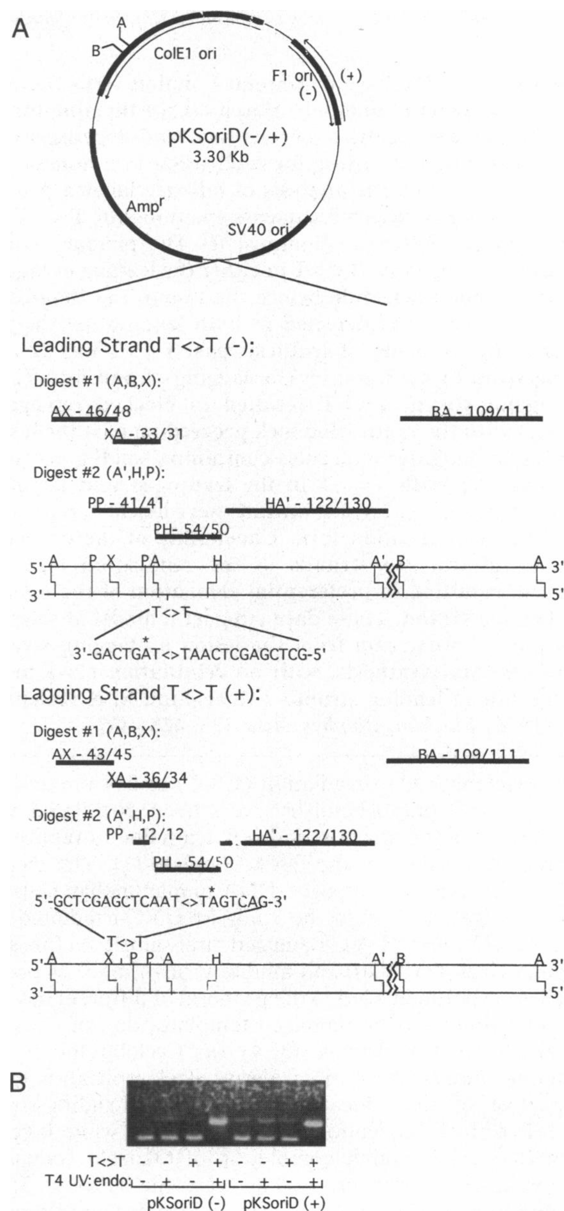

FIG. 1. T<>T-containing plasmids. (*A*) Two semisynthetic double-stranded circular DNAs 3298 nt in length, pKSoriD(−) and pKSoriD(+), and the UM counterpart pKSoriN contain the SV40 origin of replication and single site-specific T<>T dimers. The T<>T was placed in the (−) construct 342 nt from the SV40 origin in the leading template strand and in the (+) construct 357 nt from the origin in the lagging template strand. The sites of blockage of strand elongation in each strand are indicated by a star. The map of restriction sites (A = *Ase* I, X = *Xho* I, B = *Bsa* I, P = *Pvu* II, H = *Hae* II, A′ = *Afl* III) refers to those used in the sequencing gel assay (see Fig. 3*A*). (*B*) Treatment of T<>T-containing plasmids with T4 UV endo resulted in complete digestion to form II.

synthesized DNA spanning the T<>T region (Fig. 1*A*). The gel bands corresponding to each strand of each fragment were quantitated (PhosphorImager, Molecular Dynamics). Integrated intensities after background subtraction were normalized by dividing by the number of cytosine residues in each particular fragment to account for the relative incorporation of radioactive dCMP into each DNA fragment. The resulting values were taken to represent the relative molar distribution of DNA fragments produced by restriction digestion.

RESULTS

Strategy for Replication of a Strand-Specific T<>T-Containing Template. We investigated the *in vitro* replication of T<>T-containing DNA in a human cell-free extract by constructing SV40 origin-containing plasmids with a single T<>T in either the leading or lagging template strands (Fig. 1*A*). Unlabeled pKSoriD(−/+) containing the SV40 replication origin and a single, uniquely placed T<>T in either the leading (−) or lagging (+) strand treated with the Pyr<>Pyr dimer-specific T4 UV endo resulted in complete conversion of form I template DNA into nicked form II DNA (Fig. 1*B*).

Replication of T<>T in the Leading and Lagging Strands. The pKSoriD(−/+) plasmid DNAs were incubated under *in vitro* SV40 replication conditions for 10–60 min at 37°C and analyzed by agarose gel electrophoresis (Fig. 2*A*). TAg-dependent production of labeled form I DNA was observed with either leading- or lagging-strand T<>T-containing plasmids, indicating successful SV40 origin-directed replication of these modified minireplicons. There was 56% and 51% inhibition in the overall production of form I DNA, relative to UM control, when the unique T<>T was located in the leading and lagging strands, respectively (Fig. 2 *B* and *C*).

Blockage of Strand Elongation by Leading- or Lagging-Strand T<>T. DNA produced in the replication assay was treated with a combination of restriction enzymes and analyzed on polyacrylamide-sequencing gels as described in *Materials and Methods* (Fig. 3*A*). The fragments form a contiguous sequence spanning T<>T, with the exception of the late replicating, distal 109/111-bp fragment, located ≈180° from the origin (Fig. 1*A*). Blockage of strand elongation occurs 1 nt before T<>T when in either the leading or lagging template strand (Fig. 3*A*). This inhibition of strand elongation is visualized as a 28-nt fragment from the lagging-strand T<>T template and a 38-nt fragment from the leading-strand T<>T template. Full-length 46-mer and 34-mer were also detected with the modified templates, indicating that a fraction of the T<>T in the leading and lagging strands could be bypassed.

Bypass Replication of Leading- and Lagging-Strand T<>T. The conversion of form I to form II DNA by T4 UV endo confirmed synthesis of form I DNA from DNA molecules containing T<>T in the leading or lagging strand (Fig. 2*A*). Quantitation of nicks produced by treatment of replication products with T4 UV endo can be used to determine the efficiency of bypass replication of T<>T in the leading and lagging strands (see Eq. **1** in *Materials and Methods*). Replication of the damaged plasmids proceeds at 22 ± 6% for the leading-strand T<>T and 13 ± 2% for the lagging-strand T<>T relative to UM (Fig. 2*D*).

Selective Replication of the Undamaged Strand on T<>T-Containing Template. Complete, semiconservative replication of form I DNA containing a single T<>T should result in 50% conversion of daughter form I DNA molecules to form II after treatment with T4 UV endo. Quantitation of the conversion of form I DNA to form II by T4 UV endo for the different time points showed that only 33 ± 6% of leading strand and 12 ± 3% of lagging-strand T<>T product form I molecules contained T<>T (data not shown). Second-round replication of unmodified newly replicated molecules was eliminated as a possible contribution to the disproportionate UM form I product because replication products generated from fully methylated template DNA after 30 min in the replication assay as measured by digestion with methylation-sensitive *Mbo* I was not detected and because after 60 min of replication, only

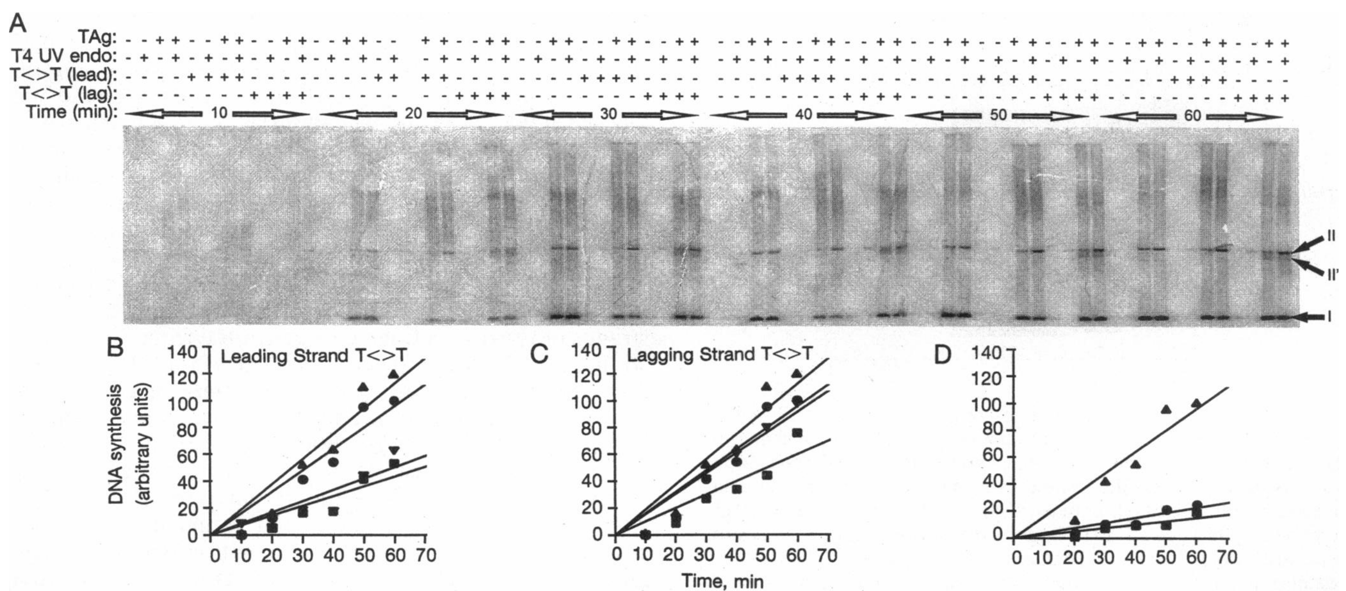

FIG. 2. Agarose gel analysis of *in vitro* replication products. (*A*) Semisynthetic plasmid DNA molecules containing a single T<>T dimer in either the leading (lead) or lagging (lag) template strand were incubated with HeLa cytoplasmic extract for 10–60 min at 37°C. After isolation of the product DNA, samples were treated with T4 UV endo at 150 ng/μl in 25 mM NaH_2PO_4/Na_2HPO_4 (pH 6.8) containing 10 μg of bovine serum albumin per ml for 30 min at 37°C and were electrophoresed on a 0.8% agarose gel containing ethidium bromide. Labeled form I DNA containing T<>T is nicked by T4 UV endo and migrates as form II. (*B*) Comparison of synthesis using leading-strand T<>T versus UM template. ●, Form I UM; ▲, form I and form II UM; ■, form I leading-strand T<>T; ▼, form I and form II leading-strand T<>T. (*C*) Comparison of synthesis using lagging-strand T<>T versus UM template. ●, Form I UM; ▲, form I and form II UM; ■, form I lagging-strand T<>T; ▼, form I and form II and form II′ lagging-strand T<>T. (*D*) Relative rates of synthesis of form I DNA containing T<>T. ▲, Form I UM; ●, form I leading-strand T<>T; ■, form I lagging-strand T<>T.

10–18% was digested (data not shown). We propose that, while synthesis is blocked on the T<>T-modified template strand, synthesis may continue on the undamaged template strand up to completion. With T<>T in the leading strand, an average of 33% of product molecules were T4 UV endo-nicked, indicating that 34% of the form I product resulted from the selective completion of synthesis on the undamaged lagging strand. For the lagging-strand T<>T template, only 12% of the form I product was nicked, indicating that 76% resulted from the selective completion of synthesis on the undamaged

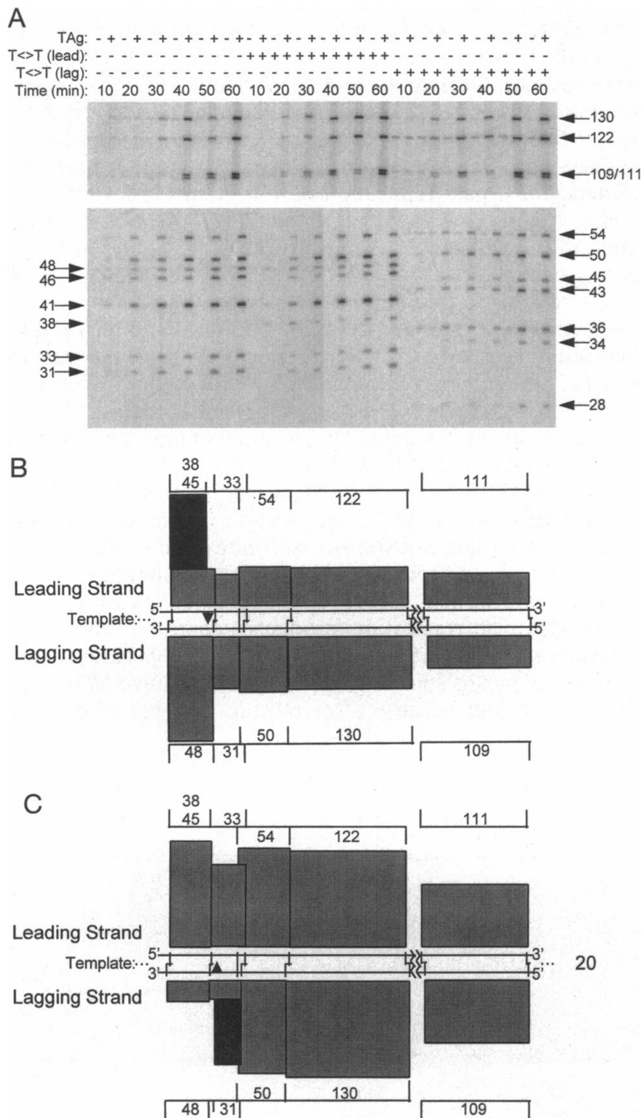

FIG. 3. Sequencing gel analysis of replication products digested with restriction enzymes. (*A*) Autoradiograph of an 8% sequencing gel showing restriction fragments obtained from a time-course replication assay. Numbers beside the gel indicate the size of the fragment in nucleotides (see Fig. 1*A*). Labeled DNA from a replication assay, as described in the legend to Fig. 2, was digested with restriction enzymes selected to yield fragments containing and flanking T<>T. Restriction enzymes were chosen so that each strand of each fragment could be resolved and analyzed on polyacrylamide sequencing gels. (*B* and *C*) The relative intensity of synthesized DNA (20-min time point) is plotted on a map of the template with restriction enzyme cut sites shown along with fragment size. The upper strand represents the synthesized leading strand and the lower strand represents the lagging strand. The darker blocks show the intensity of the blocked fragments. Two restriction digests were carried out, each with three different enzymes: Leading-strand T<>T in *B* is indicated by ▼, and lagging-strand T<>T in *C* is indicated by ▲. Digest 1 (*B*), *Ase* I/*Xho* I/*Bsa* I; Digest 2 (*C*), *Hae* II/*Pvu* II/*Afl* III. (See also Fig. 1*A*.)

Table 1. Differential replication of T<>T in leading and lagging strand

Replication product	Differential T<>T replication, %	
	Leading strand	Lagging strand
Bypass replication*	22 ± 6	13 ± 2
Uncoupling (leading)†	22 ± 10	—
Gap formation (lagging)†	—	36 ± 12
Inhibition (blockage)‡	56 ± 8	51 ± 6

The rate of synthesis of a replication product is obtained from the slope of a plot of the background-corrected intensity of the appropriate gel band vs. time as determined by PhosphorImager (Molecular Dynamics) autoradiography and is expressed relative to the rate of synthesis of form I DNA with UM control template. The average from three experiments ± SD is given.
*From Fig. 2*D*.
†See Eq. **2**.
‡Inhibition = 100 − % uncoupling (or % gap formation) − % bypass.

template strand. In other words, the rate of accumulation of excess non-damage-containing product was 22% for the leading-strand T<>T and 51% for lagging-strand T<>T (Table 1). The mechanism generating these partially replicated T<>T-containing molecules is different when T<>T is in the leading and lagging strands.

Fork Uncoupling with Leading-Strand T<>T. Comparison of the relative level of synthesis of complementary strands for restriction fragments spanning T<>T (Fig. 3 *B* and *C*) suggests a mechanism for the interaction of a replication fork with T<>T. At early time points, the reciprocal replication fork has not converged on the replication fork stalled at T<>T, indicated by the low level of incorporation of label into the distal 109/111-bp fragment (located ≈180° from the origin). With leading-strand T<>T plasmid, the relative intensities of the various fragments downstream from the lesion show that blockage occurred on the T<>T-modified leading-strand template as well as on the undamaged lagging-strand template (Fig. 3*B*). Thus, the replication fork was stalled on both strands. However, the ratio of the level of synthesis of the lagging-to-leading strands downstream from T<>T was 1.5 ± 0.1, instead of the expected equimolar synthesis of both strands. In other words, 50% excess synthesis on the lagging strand occurred while synthesis on the leading strand was blocked at the T<>T site. This observation, in agreement with the excess production of non-dimer form I molecules from the leading T<>T plasmid, indicates that there is uncoupling of the coordinated synthesis of the two strands in a fraction of the replication forks encountering the lesion in the leading template strand (Table 1).

Gap Formation with Lagging-Strand T<>T. In contrast to the leading-strand T<>T plasmid, the complementary strands of restriction fragments generated from the lagging-strand T<>T plasmid were synthesized in a 1:1 ratio (Fig. 3*C*). However, the synthesis of the fragment directly upstream from the T<>T site was selectively inhibited on the lagging strand. This observation indicates that the replication fork proceeds past T<>T, leaving behind a small gap extending from T<>T to the terminus of the previous retrograde initiation site (Okazaki fragment) (23). This conclusion is supported by the observation that a subpopulation of form II DNA (form II′) exists as a slightly faster migrating product of lagging-strand T<>T replication (Fig. 2*A*). We conclude that this form II′ synthesis from lagging-strand T<>T plasmid represents incompletely synthesized T<>T-containing molecules carrying a small single-stranded gap. In addition, the sum of the synthesis of form I + form II + form II′ DNA from the lagging strand T<>T template represents 88% of that obtained with UM plasmid, compared to only 44% for leading T<>T plasmid. Therefore, in contrast to the leading-strand T<>T template, there

is little inhibition of replication fork progression on the lagging-strand T<>T template.

DISCUSSION

By constructing semisynthetic SV40-based plasmid DNAs containing unique site- and strand-specific T<>T (Fig. 1), we examined the effect on replication of this UV-induced genotoxic lesion in the leading and lagging template strands of a human replication fork. We observed complete *in vitro* replicative synthesis of a single T<>T in the leading or lagging strands, using a *bona fide* bidirectional, semi-conservative DNA replication apparatus from a human source (17, 23, 24). We characterized stalled replication intermediates, which revealed different modes of replication of T<>T in leading or lagging strands, on the basis of fork uncoupling and gap formation, respectively.

T<>T Blockage and Bypass Replication in Leading and Lagging Strands. We observed blockage of strand elongation by the T<>T-containing template when either the leading or lagging strand was damaged. In both cases, the primary site of blockage occurred directly 3′ to the T<>T sequence (Figs. 1*A* and 3*A*). This observation extends previous studies demonstrating blockage T<>T at the 3′ nucleotide when using primed single-stranded templates with purified prokaryotic and eukaryotic DNA polymerases, including DNA polymerase δ (11–13). However, blockage is not absolute, since we saw bypass replication of T<>T for both leading and lagging modified minireplicons. Specifically, the efficiency of bypass replication of T<>T in the leading strand template was higher than that with T<>T in the lagging strand (Table 1). Thus, different mechanisms of bypass replication may operate within a replication fork, for example, through the recruitment of different DNA polymerases or accessory factors. A DNA polymerase, possibly distinct from a stalled DNA polymerase δ, may complete synthesis opposite the T<>T dimer, or a post-replicative gap-filling mechanism may exist that employs a DNA polymerase distinct from that of the normal replication machinery (2).

Gapped Circular Product Molecules from Synthesis on Lagging-Strand T<>T Template. Replication of a lagging strand-specific T<>T minireplicon was observed with little inhibition of replication fork progression and formation of small gaps, presumably smaller than an Okazaki fragment, upstream from the replication fork. Retrograde synthesis of the lagging strand is blocked at T<>T, leaving a gap extending up to the terminus of the previously synthesized Okazaki fragment. With time, the replication fork proceeds, meeting the oncoming replication fork from the opposite direction, and completes replication with decatenation of the two daughter molecules. The daughter molecule synthesized from the undamaged strand is complete and migrates as form I DNA. The daughter molecule from the T<>T-containing strand possesses a small gap, $\approx$200 bp or less, and migrates as form II′ DNA. In confirmation, specific accumulation of nicked (form II) and gapped (form II′) replicated products was observed with the lagging T<>T replicon and not the leading T<>T replicon (Fig. 2*A*). Thus, gapped synthesis with reinitiation downstream from the lesion can occur on the lagging side of the replication fork.

Fork Uncoupling with Leading-Strand T<>T Template. Although elongation of both strands at the replication fork was stalled by leading-strand T<>T, the synthesis of the lagging strand appeared uncoupled from the stalled leading strand. Preferential production of replicated molecules from the undamaged lagging strand was observed in both the T4 UV endo and restriction endonuclease assays. If both strands are synthesized by the coordinated action of two molecules of DNA polymerase δ (25), this study indicates that concerted synthesis by a dimeric DNA polymerase δ complex can be uncoupled in particular situations such as a blocking lesion on the leading side of the replication fork. If so, this model would predict the existence of stretches of a single-stranded region of DNA downstream from the damage site in the leading strand. Intriguingly, single-stranded DNA regions along human chromosomes have been reported, although their origins and fates remain unclear (26, 27).

We have demonstrated the complete SV40 origin-initiated *in vitro* replication of T<>T-containing DNA in a human cell extract. Specifically, the comparison of the fate of a *bona fide* replication fork stalled at T<>T in either the leading or lagging strand led to the conclusion that reinitiation occurs on the lagging strand but not on the leading strand. This observation supports the early model proposed by Meneghini and Hanawalt (7), where lesion-induced blockage occurs in the leading strand and reinitiation downstream from the blockage occurs in the lagging strand. However, our study also highlights the existence of fork uncoupling, permitting the selective elongation of the undamaged lagging strand in the presence of a T<>T-blocked leading strand. Thus, the recovery processes of damage-induced blockages differ significantly between the leading and lagging strands, requiring different levels of strand completion and gap filling.

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