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Pyrimidine Dimers Block Simian Virus 40 Replication Forks

CHERYL A. BERGER† AND HOWARD J. EDENBERG*

Department of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46223

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UV light produces lesions, predominantly pyrimidine dimers, which inhibit DNA replication in mammalian cells. The mechanism of inhibition is controversial: is synthesis of a daughter strand halted at a lesion while the replication fork moves on and reinitiates downstream, or is fork progression itself blocked for some time at the site of a lesion? We directly addressed this question by using electron microscopy to examine the distances of replication forks from the origin in unirradiated and UV-irradiated simian virus 40 chromosomes. If UV lesions block replication fork progression, the forks should be asymmetrically located in a large fraction of the irradiated molecules; if replication forks move rapidly past lesions, the forks should be symmetrically located. A large fraction of the simian virus 40 replication forks in irradiated molecules were asymmetrically located, demonstrating that UV lesions present at the frequency of pyrimidine dimers block replication forks. As a mechanism for this fork blockage, we propose that polymerization of the leading strand makes a significant contribution to the energetics of fork movement, so any lesion in the template for the leading strand which blocks polymerization should also block fork movement.

A variety of chemical and physical agents damage DNA within cells, causing inhibition of DNA replication and transcription, alteration of gene expression, mutagenesis, carcinogenesis, or cell death. Inhibition of DNA replication, when it occurs, is crucial to the fate of the cell: although cells may tolerate some mutations, they must replicate their full complement of DNA before dividing. The structures that result from attempting to replicate a damaged DNA template are the substrates for subsequent repair and recovery processes. Understanding the initial interaction between lesions and the replication fork will therefore provide insight into the mechanisms of recovery.

UV light is one of the best-studied of DNA-damaging agents. The primary lesion produced by UV irradiation is the *cis-syn* cyclobutane pyrimidine dimer (31). The mechanism by which UV inhibits eucaryotic DNA replication remains controversial, especially the question of whether dimers inhibit all, most, or no replication forks. The earliest model (16) proposed that a pyrimidine dimer in the template blocked elongation of a daughter strand without affecting the progression (unwinding and movement) of the replication fork, which reinitiated synthesis about 1,000 nucleotides downstream, leaving a long single-stranded gap that could later be filled de novo (Fig. 1B). This resembles a situation in *Escherichia coli*, except that in *E. coli* the gaps are filled by recombination (23, 24). An alternative hypothesis (7) is that lesions block the movement of the replication fork itself, as in Fig. 1C. With increasing awareness of the semidiscontinuous nature of DNA replication in mammalian cells (Fig. 1A), the latter model was modified to suggest that lesions in the template for the continuously synthesized (leading) strand block replication fork progression (Fig. 1C), whereas lesions in the template for the discontinuously synthesized (retrograde) strand merely block completion of an Okazaki fragment (Fig. 1D) and therefore leave small gaps averaging half the size of an Okazaki fragment (18). Alternatively, a block on either side of the replication fork might halt the entire fork (Fig. 1F), e.g., if synthesis of both daughter strands occurred in a single macromolecular complex (1). These models predict different structures in the newly replicated DNA. Lesions could be in fully double-stranded DNA (bypass; Fig. 1E), near the end of a single-stranded gap (Fig. 1B and D), or near the apex of a replication fork (Fig. 1C and F), perhaps with the entire replisome still in place. The different lesions therefore predict different substrates and constraints on subsequent repair or recovery processes.

To study the mechanism by which UV light inhibits DNA replication, we examined the effects of UV light on the replication of simian virus 40 (SV40) in monkey cells. SV40 is a small double-stranded DNA virus that replicates in the nucleus, has a chromatin structure very similar to that of the host cell, and uses host enzymes for its bidirectional replication (28). In essentially all ways measured, events at the SV40 replication fork mimic those of the host cell (6, 12). It thus provides an excellent model of a single mammalian replicon. Its small size, its well-characterized replication pattern, and our ability to extract replicating molecules make it a favorable system to study.

Previous studies, including our own, have found that UV light inhibits the overall rate of SV40 DNA synthesis, as measured by incorporation of tritiated thymidine (3, 9, 25, 26, 29, 30). The completion of replication (closing the final phosphodiester bond to make covalently closed circular [form I] daughter molecules) is even more severely inhibited (3, 9, 25, 26, 30). Daughter strands made after UV irradiation are approximately equal in length to the distance between dimers on the template DNA (3, 9, 25) as expected if the elongation of DNA is blocked at the sites of lesions, as has been found in vitro (20).

There is an additional important question: is the progression of the replication fork itself blocked by UV lesions? DNA fiber autoradiography of mammalian cells is consistent with blockage of replication forks (4, 8, 10). Most data on SV40 replication are also consistent with the blocking of replication fork progression by pyrimidine dimers in the template strand (3, 9, 19, 25, 26) but cannot completely rule out blocking strand elongation only. White and Dixon (29), however, interpreted their results as showing that replication forks neither pause nor stop at lesions but rather progress at the normal rate, leaving only a small gap opposite the lesion.

* Corresponding author.

† Present address: Cheryl A. Berger, Department of Dermatology, Oregon Health Sciences University, Portland, OR 97201.

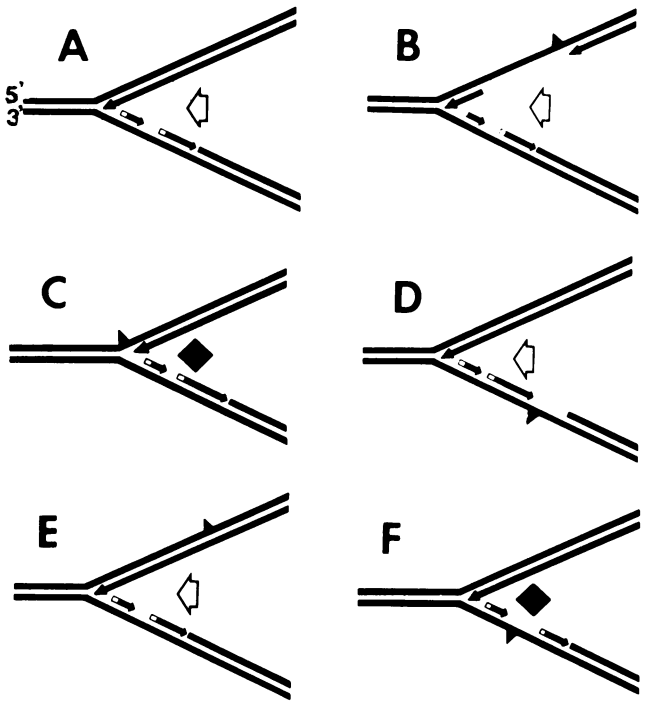

FIG. 1. Models of replication. Orientation of all panels is the same. (A) Mammalian replication fork, semidiscontinuous replication. Leading strand synthesized from right to left. (B) Continuous-strand gaps. Lesions in the template for the continuous strand cause termination of daughter strand elongation but fork progression continues uninterrupted, and synthesis of the halted daughter strand resumes some distance downstream. (C) Semidiscontinuous blockage. Lesions in the template for the continuous strand block not only strand elongation but also fork progression and therefore also stop further synthesis of the opposite strand; no long gaps are left. (D) Semidiscontinuous blockage. Lesions in the template for the retrograde strand merely block completion of a single Okazaki fragment without blocking fork progression, leaving a small gap. (E) Bypass. Lesions on either strand are continuously bypassed without leaving gaps. (F) Coupled synthesis. Lesions on either strand block replication fork progression. Symbols: ▲, lesion (e.g. pyrimidine dimer); open arrowhead, replication fork continues to move; ◆, replication fork blocked.

Their interpretation seems inconsistent with data showing that most label incorporated after UV irradiation is near the origin of replication, as if dimers block fork progression (25). Also, it is difficult to account for the observed rapid onset of a profound inhibition of replication (9) without invoking at least a significant delay at the site of a lesion.

We directly tested whether lesions block replication fork progression, as opposed to merely blocking elongation of a daughter strand while the fork skips past and reinitiates. We isolated replicating SV40 molecules and analyzed by electron microscopy the positions of the replication forks. We found that dimers in the template for the leading strand blocked the movement of replication forks, and we propose a mechanism for this blockage.

MATERIALS AND METHODS

Cells, virus, and labeling. Protocols and preparative methods were as previously reported (9). CV-1 cells were infected with SV40 (strain 776) and irradiated with UV light from a 15-W germicidal lamp (Sylvania G15T8) at 40 h postinfection, when viral replication is at a maximum rate. The DNA from a radioactively labeled 60-mm dish was mixed with that from a nonradioactive 150-mm dish treated in parallel, so that most molecules were not radioactive. Samples were dialyzed against TEN (10 mM Tris chloride [pH 8.0], 10 mM NaCl, 1 mM EDTA) for 3 to 5 h at 0°C and centrifuged to equilibrium in CsCl gradients containing 400 μg of ethidium bromide per ml. A narrow form I pool and a broad replication intermediate (RI) pool that extended from the form I pool to the far side of the form II (nicked circular molecules) were selected (9) to avoid biases for or against nicked molecules in the RI pool. Benzoylated-naphthoylated DEAE-cellulose chromatography was performed as previously described (11).

Alkaline sedimentation of DNA. Samples of the original viral DNA extract were ethanol precipitated, suspended in TEN, adjusted to 0.2 M NaOH, and sedimented (90 min, 49,000 rpm, 20°C in a Beckman SW50.1 rotor) through gradients from 5 to 20% sucrose in 0.2 M NaOH–0.8 M NaCl–2 mM EDTA.

Dimers per molecule. Under the irradiation conditions used here, the number of dimers introduced is linear with fluence to at least 16 dimers per SV40 molecule (22). Each 10 J of UV light per m^2 introduces an average of one pyrimidine dimer per SV40 molecule (9). The same number of dimers is introduced into all forms of SV40 (chromatin, previrions, and virions) within cells (13).

Electron microscopy. Samples of SV40 RI DNA, purified on CsCl gradients and by benzoylated-naphthoylated DEAE-cellulose chromatography, were digested for 40 min with *BglI* (Bethesda Research Laboratories, Inc.). The reaction was stopped with 20 mM EDTA, and proteins were extracted with chloroform-isoamyl alcohol (24:1, vol/vol). DNA was mounted from a hyperphase of 40% formamide onto a hypophase of 10% formamide, stained, and shadowed, all as described by Davis et al. (5). Grids were scanned methodically, and all potential RIs were photographed at a magnification of ×20,000 in a Zeiss 109 electron microscope; this included any molecule not easily traced as linear (excluding small fragments or extremely long or tangled pieces). This protocol was chosen to minimize bias in selection of molecules, at the cost of photographing many that proved on closer examination not to be RI. Negatives were printed at a final magnification of approximately ×100,000 and measured by tracing the arms of each molecule twice on a digitizing tablet (Apple Graphics Tablet) attached to a microcomputer (Apple II Plus). Lengths are in the arbitrary units generated by the digitizing tablet (1 U = 30 base pairs [bp]).

Data analysis. We initially measured and tabulated all H-shaped molecules from each preparation. Among these were several abnormally short or long molecules that do not represent replicating SV40 (e.g., fragmented cellular or viral DNA). To avoid bias in excluding these, we chose a statistical procedure. We determined that the total length of the H-shaped RIs from our largest control population ($N = 123$) was 170.8 ± 10.9 U (mean ± standard deviation); no preparation differed significantly ($P > 0.5$ by Student's t test). We then excluded all molecules in any preparation whose length differed from this mean by more than two standard deviations; about 10% of the molecules were excluded, most of which were far larger or smaller than the cutoff.

To determine the distance of each replication fork from the origin, we averaged the lengths of the two branches at that fork. We determined the difference in the average

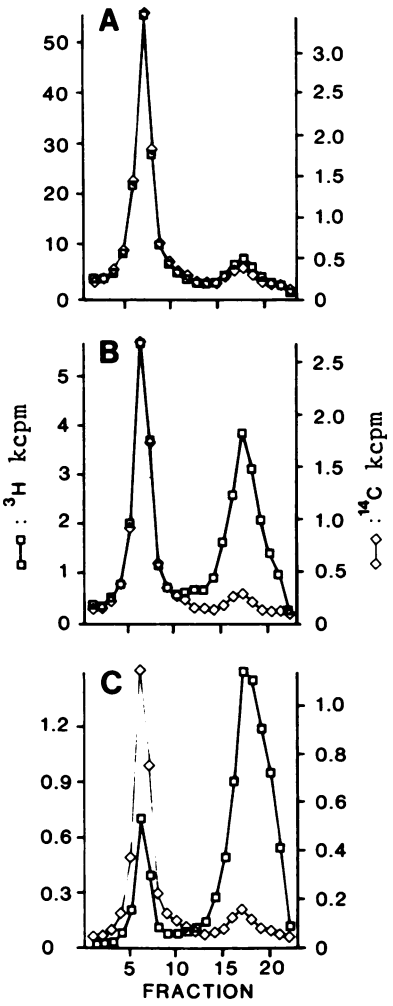

FIG. 2. Alkaline sedimentation of DNA synthesized after UV irradiation. Parental SV40 DNA was prelabeled with [^{14}C]dT, the cells were irradiated, and DNA made during the first 90 min after UV was labeled with [^{3}H]dT. Samples were prepared and sedimented as described in the text; sedimentation is from right to left. The left peak is form I DNA, the right peak includes form II and RI. (A) 0 UV; (B) 1.2 dimers per molecule; (C) 3.6 dimers per molecule.

position of the two forks within the same molecule ($b - a$; see Fig. 4), rather than the ratio, to avoid compounding errors of measurement.

We found some H-shaped molecules in which one branch at a single fork was longer than the other (i.e., $a \neq a'$ or $b \neq b'$; see Fig. 4). We estimated the contribution of random errors of measurement to the difference in branch length by calculating the difference at each fork in the control sample (2.8 ± 2.4 U [mean ± standard deviation]). We then assigned to the second category all molecules in which the difference in branch length at either fork exceeded 7.5 U (broken branches). This group of molecules was handled separately (see Results) and not plotted in Fig. 6 and 7, although their inclusion would not alter the patterns shown.

RESULTS

DNA synthesis after UV irradiation. UV irradiation inhibits incorporation of thymidine into SV40 DNA in a dose-dependent manner. An average of 1.2 dimers per molecule reduced total incorporation (during a 90-min pulse) to 16% of the control, and 3.6 dimers reduced it to 11% (Fig. 2). Of the radioactivity incorporated in the control, 80% was in completed molecules (form I, left peak in Fig. 2). By comparison, only 48% (at 1.2 dimers) and 19% (at 3.6 dimers) of the reduced incorporation was into form I (Fig. 2B and C). The inhibition of form I synthesis is dramatic: taking into account the overall reduction in incorporation and the change in the fraction of molecules completed, an average of 1.2 lesions per molecule reduces form I synthesis to 8% of control, and 3.6 lesions reduces it to 2%. Similarly dramatic inhibition has been shown even in very brief pulses (9). We have previously shown that much of the total incorporation (particularly into form I) occurs in the first few minutes after UV irradiation, as molecules that were in the process of replication at the time of irradiation are completed (9). The inhibition during long pulses also reflects packaging of DNA into virions and failure to replenish the pool of replicating molecules (22).

Analysis of benzoylated-naphthoylated DEAE-cellulose chromatography (Fig. 3) confirmed that post-UV incorpora-

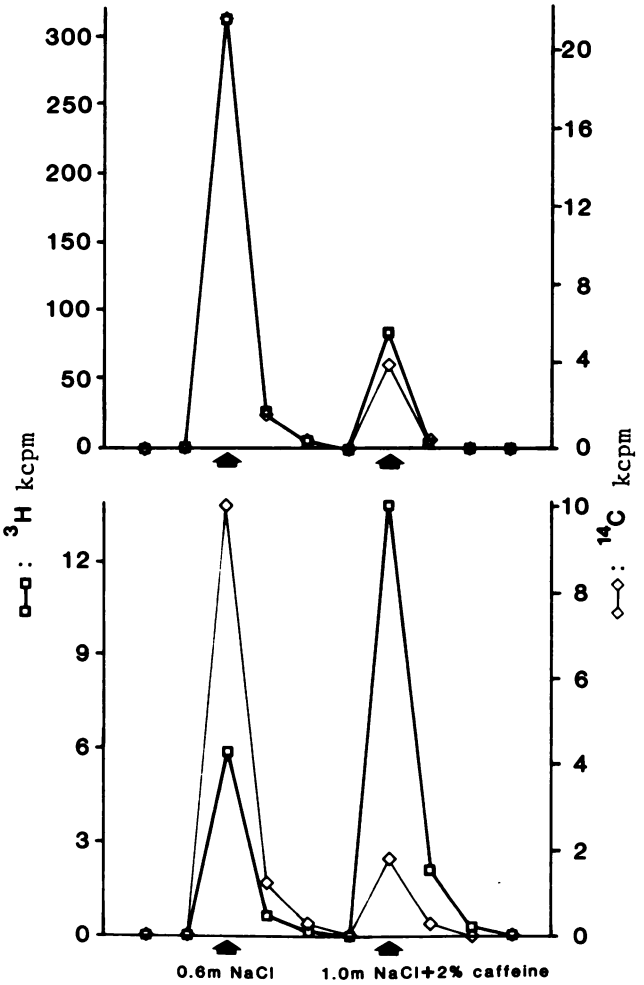

FIG. 3. Benzoylated-naphthoylated DEAE-cellulose chromatography of DNA made during the first 90 min after UV irradiation. Samples of the Hirt supernatant from the experiment shown in Fig. 2 were chromatographed as described (11). Left peak (0.6 M NaCl eluate), double-stranded DNA; right peak (1.0 M NaCl + 2% caffeine eluate), RI. (A) 0 UV; (B) 3.6 dimers.

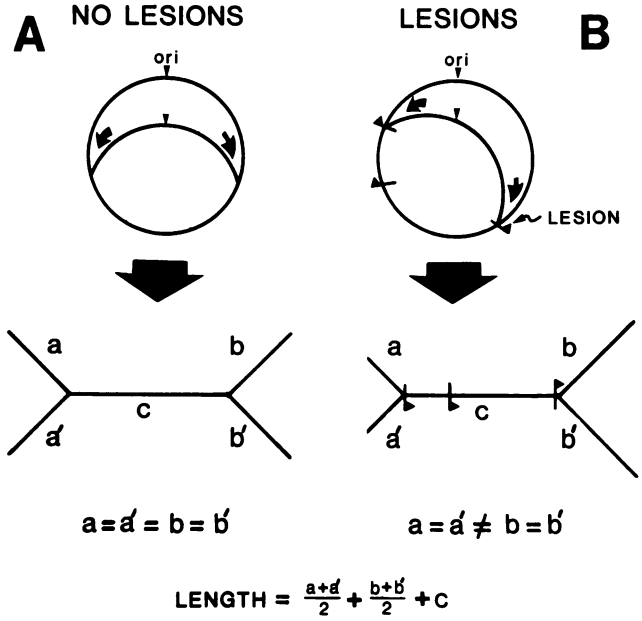

FIG. 4. Electron microscopic strategy: SV40 is a circular molecule with a fixed origin of replication (*ori*) and bidirectional replication (small arrows). Cleavage at the origin (with *BglI*) results in H-shaped molecules; the two branches at each fork are equal in length ($a = a'$; $b = b'$). (A) In molecules without lesions or molecules in which lesions do not block replication forks, the two forks progress at roughly equal rates so at any time they are equidistant from the origin ($a = a' = b = b'$). (B) Lesions (flags) are introduced at random, and therefore in any one molecule are likely to be at different distances from the origin. If they block replication forks (and not merely elongation of a daughter strand), then in most molecules the forks will be blocked at different distances from the origin ($a = a' \neq b = b'$). Total length of the molecule is determined by adding the distance traveled by both forks to the length still unreplicated.

tion is predominantly into RIs (right peak) rather than into completed form I molecules. The fraction of pulse-labeled molecules that sediments slowly in alkaline sucrose (Fig. 2) equals the fraction that behaves as an RI on benzoylated-naphthoylated DEAE-cellulose columns (Fig. 3).

Strategy of electron microscopic analysis. During normal SV40 replication, the two replication forks diverge from a fixed origin (about 30 bp from the *BglI* site) at approximately equal rates (14, 17, 27). Thus, the replication forks in a single molecule will usually be at equal distances from the origin (17, 27; see Fig. 4 and 6). Since dimers are randomly introduced, in any one molecule the distance to the nearest dimer on one side of the origin will usually differ from that on the other. If dimers block replication forks, then in a fraction of the molecules the replication forks will be blocked at different distances from the origin. In some molecules the forks will not have reached a dimer, and in others dimers may by chance lie at roughly equal distances from the origin; these classes of molecules will be symmetrical even if dimers block forks. If dimers do not block replication forks, then the fork positions should be symmetrical, as in the control, although some models (Fig. 1B) predict long single-stranded regions in the replicated DNA behind the fork.

We analyzed this by partially purifying SV40 RI, digesting it with *BglI*, and visualizing the resulting molecules. RI molecules appeared as H-shaped molecules, and the position of both forks with respect to the origin was determined (Fig. 4). We examined populations of H-shaped replicating molecules from irradiated and control cultures and compared the distance of each fork from the origin (i.e., $[a + a']/2$ versus $[b + b']/2$) in each molecule. Only if lesions block replication forks (Fig. 1C and F) do we expect to see asymmetry (one fork significantly farther from the origin than the other [Fig. 4 right] in a substantial fraction of the molecules.

Electron microscopic data: H-shaped molecules. We compared populations that had an average of 0, 5, or 7.5 dimers per SV40 molecule. Selected molecules (extracted 45 min after UV irradiation) are shown in Fig. 5. The replication forks in the unirradiated population were equidistant from the origin. The relationship of the lengths of the short arms (a in Fig. 4) versus the length of the long arms (b) is shown in Fig. 6A. Since we plotted the shorter of the arms on the ordinate, the points fell below the 45° line. The overall symmetry is apparent: in essentially all molecules at all stages of replication, $a \approx b$. The difference in fork position ($b - a$) is only 4.8 ± 3.8 U (mean ± standard deviation; see Table 1). (The units are arbitrary, determined by the graphics tablet.) The average length of an SV40 molecule is 171 U (Table 1) so this difference is less than 3%. The 30-bp difference between the *ori* and the *BglI* site (14, 17) should produce a difference of 1 U on this scale. After UV irradiation that produces an average of five dimers per molecule there is a large fraction of asymmetrical molecules (Fig. 6B and a large mean difference in fork position (30.8 ± 20.1 U, equal to 930 bp; Table 1). Molecules with an average of 7.5 dimers also show a marked asymmetry (Fig. 6C) and a mean difference in fork position of 24.8 ± 21.1 U (750 bp; Table 1). At higher numbers of lesion per molecule, the mean difference in fork position should be reduced, since lesions are closer together; this is consistent with our data (Table 1). The mean differences in fork position for both populations of irradiated molecules are significantly greater than the control ($P < 0.001$ by Student's t test). (Transforming data to the form $\log[1 + (b - a)]$ to reduce the influence of outlying molecules does not alter the significance of these differences.) Since dimers are distributed in a random (Poisson) manner, the mean and standard deviation of the distribution of distances to the dimer should be equal; our data approximate this.

The frequency distribution of molecules with different extents of asymmetry (i.e., $b - a$) is shown in Fig. 7. This confirms the symmetry of the control molecules: in 86.5% the difference in fork position is less than 8.6 U (mean difference + standard deviation). In contrast to the control, a large fraction of irradiated molecules have asymmetrical fork positions, as predicted for molecules in which UV lesions block replication fork progression: in 73% (5 dimers) and 85% (7.5 dimers), the mean difference in fork position is greater than 8.6 U. There are, as expected, symmetrical molecules among the irradiated populations. These are molecules in which dimers happen to be at similar distances on both sides of the origin, or molecules that have not yet reached a dimer.

In the unirradiated population, few molecules have long branches more than 85 U (half of the total length; in only a single molecule has the farthest fork passed the halfway point [Fig. 6A]). This is as expected, since there is no fixed termination site in SV40 (15); termination occurs where the two forks meet, usually 180° from the origin. If one fork is blocked by a dimer we predict that in some molecules the other fork should progress beyond the normal halfway point until it too is blocked (or with few lesions per molecule it may meet the other fork, leaving a gap or nick). We find in the irradiated populations many molecules in which the

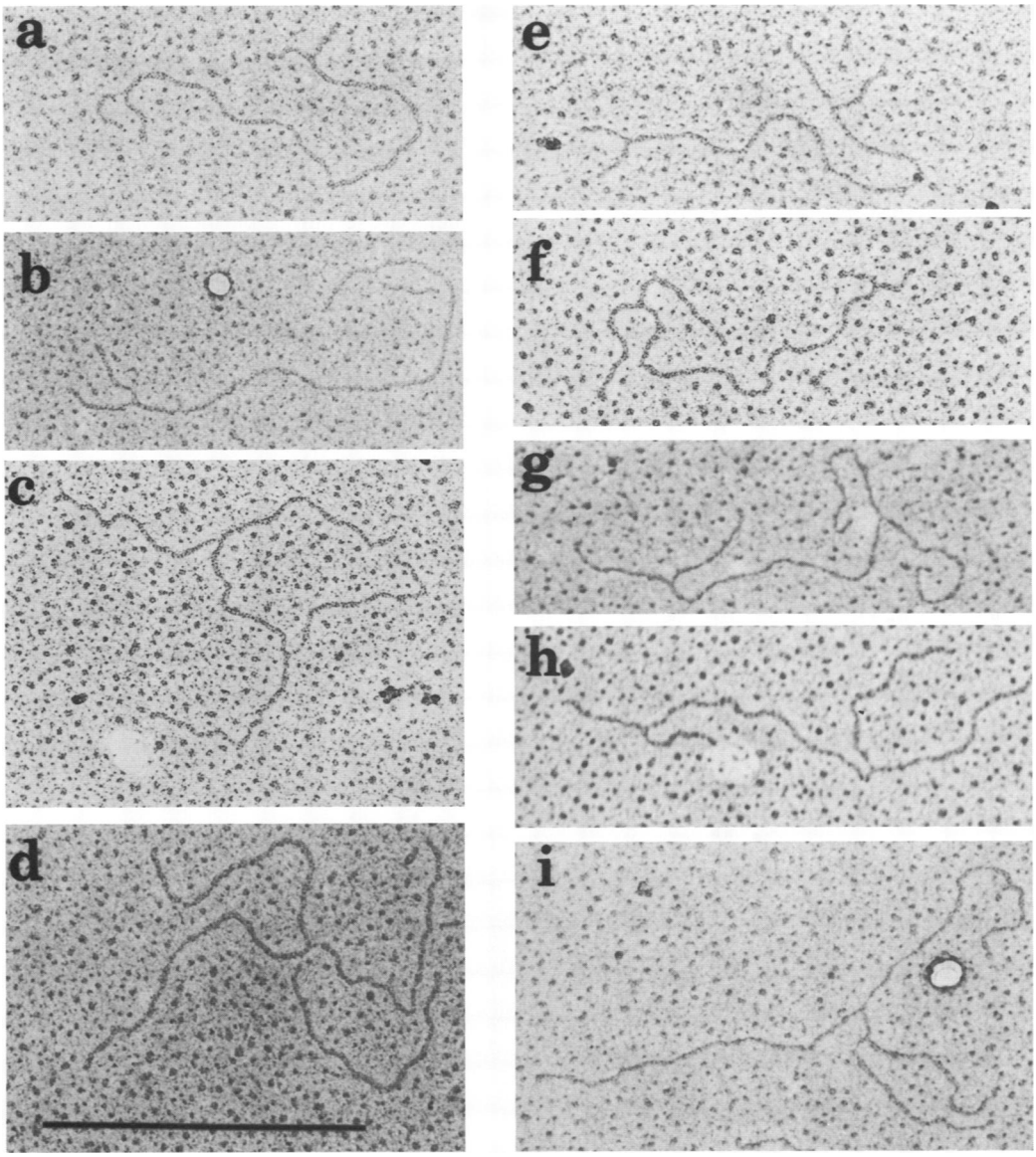

FIG. 5. Representative molecules. (a–d) Unirradiated; (e–i) irradiated to introduce an average of 7.5 dimers per molecule. Bar, 1 μm.

longer arm has passed the halfway point (9 of 27 at 5 dimers per molecule, 18 of 64 at 7.5 dimers per molecule; Fig. 6B, C), reinforcing our conclusion that UV lesions block replication forks.

Lack of single-stranded gaps. We did not observe long single-stranded regions behind the replication forks. This argues against the original Lehmann (16) model in which forks progress and reinitiate synthesis after skipping 1,000 nucleotides. Gaps of several hundred nucleotides should have been visible, since in some molecules we can visualize a small single-stranded region on one side of the replication fork, representing the short region of the retrograde strand (usually less than 200 bp) in which an Okazaki fragment has not yet been synthesized (6, 12). We might not see gaps that were smaller. Our failure to find long single-stranded regions also argues against models in which the two strands act totally independently, i.e., synthesis along one of the two strands stops while the fork progresses and synthesis continues along the other strand; this latter model predicts a very long single-stranded region (equal to the interdimer distance) immediately behind the replication fork.

The semidiscontinuous model of replication fork blockage predicts that about half of the blocked replication forks will have passed a dimer in the template for the discontinuously synthesized strand. One might therefore predict a small single-stranded gap (half the size of an Okazaki fragment, or about 50 to 150 nucleotides long) in the daughter strands. We did not directly observe these tiny gaps, which are below the detection limit of our technique, but we believe that molecules in which $a \neq a'$ or $b \neq b'$ (see below) represent molecules in which one branch broke at such a gap.

Broken molecules. We found two types of molecules that may have arisen from breakage during our preparative procedures: Y-shaped molecules and molecules in which a pair of branches at a single fork were unequal in length. There were many Y-shaped molecules in both irradiated and unirradiated populations. It is theoretically possible that they represent rolling-circle replication. However, they were

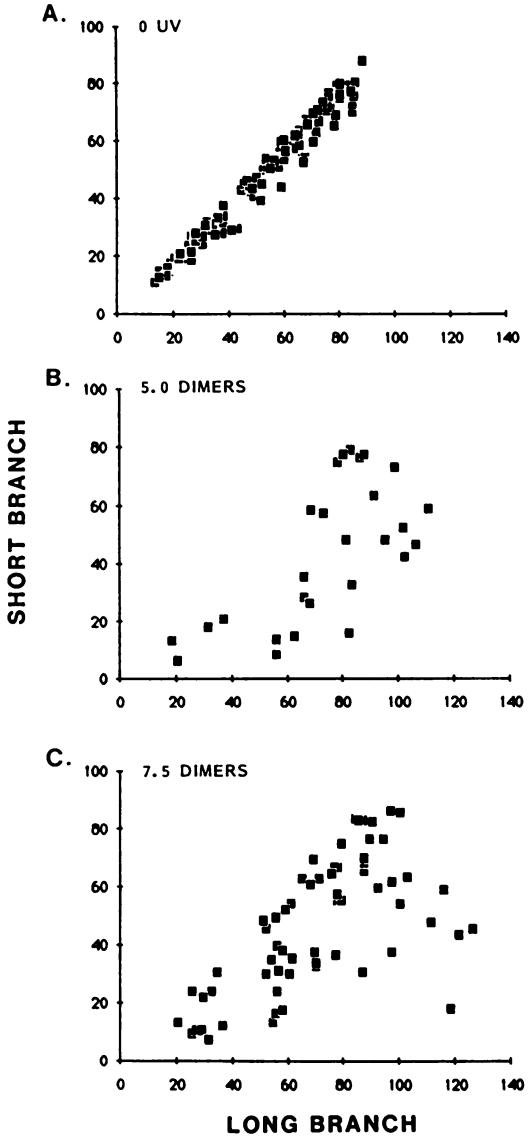

FIG. 6. Symmetry or asymmetry of fork position. Each point represents a single molecule, in which the length of the shorter pair of branches (a, distance from the origin to the nearest replication fork) is plotted versus the length of the longer pair of branches (b, distance to the farther fork). All unbroken H-shaped molecules are shown. (A) 0 UV; (B) 5.0 dimers per molecule; (C) 7.5 dimers per molecule.

present to such a large extent (1/3 to 1/2 of the potential RIs) in both control and irradiated populations that this explanation is unlikely; rolling circles generally represent fewer than 2% of the RIs (17). These Y-shaped molecules are most readily explained as a consequence of breakage at the short regions of single-stranded DNA located at the replication fork (Fig. 1A). Mezzina et al. (19) found a large fraction of UV-irradiated SV40 molecules in which one of the two replication forks was broken. Our finding is consistent with theirs: digesting (with *BglI*) a sigma-shaped molecules would yield a Y-shaped molecule. Since we find many such molecules in the control also, we favor the idea that most such nicks arose during purification and mounting of the DNA, rather than as a part of a repair process.

The second class of broken molecules was found predominantly among irradiated molecules. These were H-shaped molecules in which the two branches at a single replication fork were not of equal length (i.e., $a \neq a'$ or $b \neq b'$). In such a molecule, the length of the longest branch at each fork, rather than the average length, represents the distance

TABLE 1. H-shaped replicating molecules

No. of dimers[a]	No. of molecules	Length[b]	Difference[c]
0	111	171.4 ± 8.4	4.8 ± 3.8
5.0	27	172.4 ± 7.1	30.8 ± 20.1
7.5	64	171.1 ± 10.7	24.8 ± 21.1

[a] Average number of dimers per molecule.
[b] Length in arbitrary units, mean ± standard deviation.
[c] Difference in fork position ($b-a$), mean ± standard deviation.

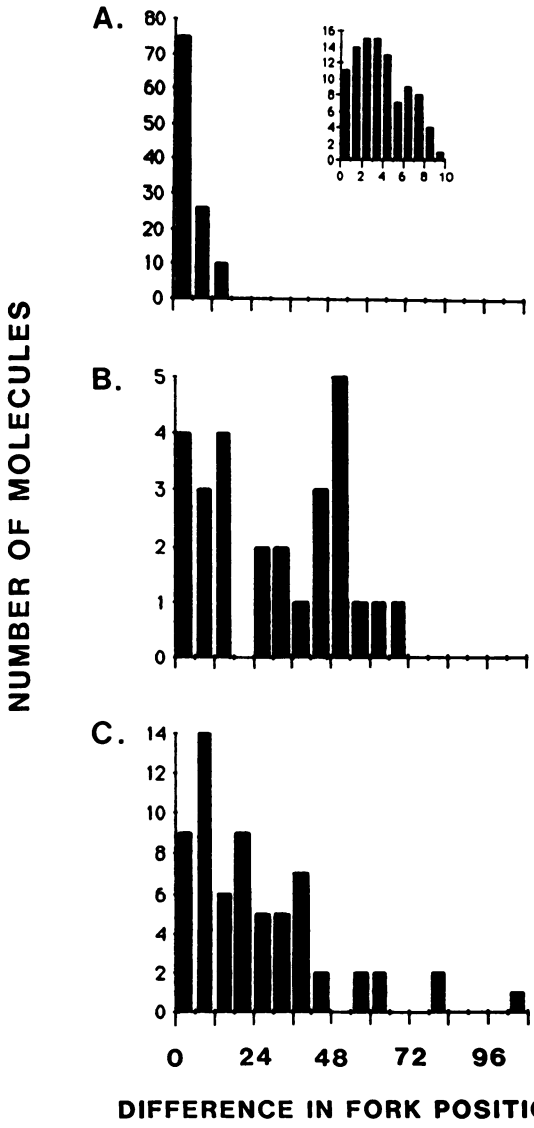

FIG. 7. Frequency distribution of differences in fork position. The difference between the position of the two forks in each molecule was calculated ($b - a$), and the number of molecules with any given difference (e.g., 0 to 5.99, 6 to 11.99) was plotted. (A) 0 UV; inset shows the region from 0 to 10 U expanded; (B) 5.0 dimers per molecule; (C) 7.5 dimers per molecule.

TABLE 2. Broken molecules

No. of dimers[a]	Shape[b]	No. of molecules	Length[c]		Difference[d]
			Raw	Corr	
0	Broken	16	166.9 ± 10.0	173.6 ± 10.1	11.1 ± 16.1
	Broken[e]	15			7.4 ± 7.0
	All	127			5.6 ± 6.9
5	Broken	28	157.2 ± 15.9	172.9 ± 9.5	27.7 ± 22.2
	All	55			29.8 ± 21.1
7.5	Broken	36	155.8 ± 17.0	173.3 ± 11.2	42.6 ± 26.8
	All	100			31.2 ± 24.7

[a] Average number of dimers per molecule.
[b] Broken, H's (i.e. $a \neq a'$ or $b \neq b'$); All = H's + broken H's.
[c] Length in arbitrary units, mean ± standard deviation. Raw, average of broken and unbroken arms at each fork; corr, the longer of the two arms at a fork.
[d] Difference in fork position ($b - a$) in arbitrary units, mean ± standard deviation.
[e] A single molecule with a very large difference (66 U; possibly the result of a defective replication fork) contributed excessively to the average of 16, so an average excluding this molecule is also shown. "All" includes this molecule.

traveled by that replication fork. If this analysis is correct, the total length of broken molecules calculated by using the longer of the two branches should equal that of the unbroken molecules, whereas the calculation made by using the average branch length should be too low; our data show that this is the case (Table 2). The data from these broken molecules are consistent with those from unbroken H-shaped molecules from the same pools. There were few broken H's among the unirradiated molecules, most of which were symmetrical (Table 2). There were many broken H's among the irradiated molecules, and these were largely asymmetrical: 71% (5 dimers) and 86% (7.5 dimers) of these irradiated molecules had a difference in fork position greater than 8.6 U, and the mean differences were comparable to those in the unbroken H's (Table 2).

DISCUSSION

Our electron microscopic data demonstrate that UV irradiation blocks replication fork progression. In unirradiated SV40, replication forks are symmetrically distributed around the origin of replication (Fig. 5–7; Tables 1 and 2), showing that the two forks move at approximately equal rates (17, 27). UV dramatically changes that situation: a very large proportion of the replicating molecules are asymmetrical (Fig. 5–7; Tables 1 and 2), indicating that at least one of the forks has been blocked. This is seen at an average of 5 or 7.5 dimers per molecule in both the H-shaped and broken molecules (and at 6 and 10 dimers per molecule in preliminary experiments; data not shown). The presence of blocked forks rules out the rapid skipping past UV lesions proposed by White and Dixon (29), which would result in symmetrical fork positions with small gaps left in the DNA. The distribution of post-UV label predominantly around the origin of replication (25) also rules out rapid resumption of synthesis beyond the dimers.

These electron microscopic data can be taken together with earlier data to provide very strong support for a model in which dimers block replication forks in a semidiscontinuous manner (Fig. 1C and D). Nascent strands made in the first hour or two after irradiation grow to a size equal to the interdimer distance on the template strand (3, 9, 19, 25). By itself, this size cannot distinguish between models in which a lesion in the template for one of the strands (e.g., the leading strand) blocks synthesis of both daughter strands by blocking fork progression and models in which daughter strand elongation is completely independent along the two strands (see reference 10 for a more detailed discussion of the predicted synthesis with different models). It does, however, rule out models in which a dimer on either strand halts the synthesis of both daughter strands, since that is statistically equivalent to having twice the number of dimers (each dimer blocks two strands) and therefore the size of the daughter strands would be half the interdimer distance (10). The electron microscopic data reported here demonstrate that UV lesions block replication fork progression and not merely elongation of a daughter strand. Since stopping fork progression necessarily blocks synthesis of both strands at that fork, the two strands cannot be completely independent. Putting these data together, we conclude that dimers in one of the two parental strands block fork progression whereas dimers in the other do not. The semidiscontinuous model in which dimers in the template for the leading strand block fork progression and dimers in the opposite strand merely block completion of an Okazaki fragment is therefore most reasonable.

Further support for this model can be obtained from a comparison of the frequency of lesions with the frequency of blocked forks. One can calculate the expected frequency distribution of dimers per molecule at any given average dimer content (randomly introduced dimers follow a Poisson distribution) and from that distribution calculate the expected fraction of molecules in which both forks could be blocked. Based on the semidiscontinuous model, the circular SV40 molecule would have both forks blocked only if at least two lesions were situated in *trans* on the templates for continuous strand synthesis. Fewer lesions or a different spatial distribution would allow at least one fork to continue until it reached the other, leaving a molecule with a small gap instead of an H-shaped replicating molecule. The situation is identical to that described for formation of long-lived unreplicated regions between converging mammalian replication forks (21). At 5 or 7.5 dimers per molecule, 71 and 89% of the molecules could potentially have both forks blocked. The fraction of asymmetrical molecules we observed (73 and 85% of the H-shaped molecules, 71 and 86% of the broken H's) is close to this, lending independent support to the model of semidiscontinuous fork blockage. This analysis explains why White and Dixon (29) did not detect a significant fraction of blocked replication forks and hence their conclusion that replication rapidly passes dimers without pausing. They used UV fluences that result in only one dimer per SV40 molecule. In a population with an average of one dimer per molecule, only 9% of the molecules would have two dimers in *trans* on the templates for the leading strand and could potentially display asymmetrically blocked forks. (37% are undamaged, and the others would result in molecules with a small nick or gap, the predominant molecules detected by White and Dixon [29].) Thus, their failure to find a significant fraction of asymmetrical molecules is expected and in no way argues against the hypothesis that dimers block replication forks.

We propose a molecular mechanism for replication fork blockage by lesions such as pyrimidine dimers; this mechanism explicitly predicts semidiscontinuous blockage. It is based on analogy to bacteriophage T4 replication, in which the two sides of the replication fork are coupled in a multicomponent "replication machine," and polymerases, helicases, and DNA-binding proteins all contribute toward

moving the T4 replication fork (1). We propose that in eucaryotic cells polymerization of the leading strand makes a significant contribution to the movement of the replication fork; therefore, any lesion that halts polymerization of the leading strand will remove one of the forces that move the replication fork forward and will thereby block fork progression. Lesions on the template for the retrograde strand would merely halt synthesis of the Okazaki fragment then being synthesized. For the fork to continue moving beyond a lesion in the template for the retrograde strand, the polymerase complex synthesizing the retrograde strand must dissociate from the blocked site and initiate synthesis of the next Okazaki fragment. We note that if cells differ in the relative contribution that polymerization makes to the progression of the replication fork, they differ in the extent to which a dimer or other lesion reduces the ability to move the fork. The balance of forces that normally move the replication fork, as well as changes in the intracellular environment (perhaps induced by the damage) that affect DNA structure and unwinding, would thus influence the interaction between lesions and replication fork progression.

Stacks et al. (26) reported that some dimer-containing molecules become fully replicated and argued that the replication machinery can accommodate a limited number of lesions. The number of molecules completed is dramatically reduced (in agreement with our data, above) so the accommodation must be quite limited. Most form I molecules that do accumulate after irradiation were partly synthesized before UV irradiation and had no lesions ahead of the replication forks (9). The suggestion of Stacks et al. that the decline in SV40 replication after UV irradiation is caused by an accumulation in the replication pool of molecules with levels of damage greater than can be tolerated is difficult to reconcile with the data since, at the low levels of UV they used, very few molecules have more than two or three lesions (only 8% of a population with an average of one lesion per molecule have more than two lesions). This small fraction of molecules could hardly explain the rapidity and severity of inhibition if one or two lesions are often accommodated. Furthermore, Barnett et al. (2) mathematically modeled an experiment similar to that of Edenberg (9) and concluded (as we did) that even a single dimer prevents completion of a form I molecule.

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