Replication of Simian Virus 40 DNA after UV Irradiation: Evidence of Growing Fork Blockage and Single-Stranded Gaps in Daughter Strands

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The molecular mechanisms of in vivo inhibition of mammalian DNA replication by exposure to UV light (at 254 nm) was studied in monkey and human cells infected with simian virus 40. Analysis of viral DNA by electron microscopy and sucrose gradients confirmed that the presence of UV-induced lesions severely blocks DNA synthesis, and thus the conversion of replicative intermediates (RIs) into fully replicated form I DNA is inhibited by UV irradiation. These blocked RI molecules present several special features when visualized by electron microscopy. (i) In excision repair-proficient monkey and human cells they are composed of a double-stranded circular DNA with a double-stranded tail whose size corresponds to the average interpyrimidine dimer distance, as determined by the dimer-specific T4 endonuclease V. (ii) In excision repair-deficient human cells from patients with xeroderma pigmentosum, UV-irradiated RIs present a Cairns-like structure similar to that observed for replicating molecules obtained from unirradiated infected cells. (iii) Single-stranded gaps are visualized in the replicated portions of UV-irradiated RI molecules; such regions are detected and clearly distinguishable from double-stranded DNA when probed by a specific single-stranded DNA-binding protein such as the bacteriophage T4 gene 32 product. Consistent with the presence of gaps in UV-irradiated RI molecules, single-strand-specific S1 nuclease digestion causes a shift in their sedimentation properties when analyzed in neutral sucrose gradients compared with undamaged molecules. These results are in agreement with and reinforce the model in which UV lesions are a barrier to the replication fork movement when present in the template for the leading strand; when lesions are in the template for the lagging strand they inhibit synthesis or completion of Okazaki fragments, leaving gaps opposite the lesion. Moreover, cellular DNA repair-linked endonucleolytic activity may induce double-stranded breaks in the blocked region of the replication forks, resulting in the tailed structures observed in viral DNA molecules obtained from excision repair-proficient cell lines.

Pyrimidine dimers are the major lesions induced on DNA by exposure to UV light at 254 nm. When damaged chromosome is not completely repaired by the various cellular repair mechanisms, these lesions may cause a wide spectrum of cellular events, including inhibition of transcription and DNA replication. In bacteria, the inhibition of DNA replication by such lesions induces the coordinated expression of several functions: the SOS system controlled by the *recA-lexA* pathway, the lysogenic induction of λ bacteriophage, Weigle's reactivation, mutagenesis, and filamentous growth (34). In mammalian cells, unrepaired dimers on the chromosome can be converted into fixed genomic mutations (15) and give rise to such other physiological consequences as alteration of gene expression, cell transformation, and carcinogenesis. Among all bulky lesions inhibiting DNA synthesis, pyrimidine dimers are the best-known lesions affecting the structural integrity of genetic material (16). However, the molecular mechanism of the inhibition of DNA replication by UV-induced lesions is still not fully understood.

Studies on the inhibition of DNA synthesis in vitro, using appropriate templates carrying pyrimidine dimers or other DNA lesions induced by DNA-damaging agents and purified bacterial or mammalian replicating enzymes, demonstrated that DNA polymerases stop synthesis one nucleotide before or at the lesions (23, 24). Earliest studies on DNA replication

after UV irradiation in *Escherichia coli* have shown that discontinuities were produced in daughter strands especially in excision-deficient bacterial strains due to the blockage of the replication fork and reinitiation of DNA synthesis beyond the lesion (26). However, the mechanisms of interactions between a mammalian replicon and such lesions during in vivo DNA replication is still a matter for speculation. Among the different models proposed for the inhibition of a mammalian replicon by UV-induced lesions (for a review, see Fig. 1 in reference 3), the more widely accepted model supposes that lesions located in the DNA template for the leading strand may block or temporarily halt fork progression, whereas lesions located in the template for the lagging strand would not impede fork movement. In this case, the completion of synthesis of an Okazaki fragment is inhibited, giving rise to a gap. These gaps would be eventually filled by cellular repair enzymes, allowing daughter molecules to start a new replication round. This hypothesis, initially proposed by R. Meneghini, was supported by biochemical analysis in human fibroblasts of newly synthesized DNA strands after UV irradiation followed by S1 endonuclease digestion (9, 21). However, analysis of cellular DNA after UV irradiation gives results difficult to interpret due to the complexity of mammalian chromosomes and to the existence of multiple replicons. Simian virus 40 (SV40) presents an elegant alternative model for these studies. This double-stranded DNA virus consists of a single replicon unit which basically mimics DNA replication and thus the fork movement of the host chromosome (5). Using this virus as a probe for

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studying the replication of UV-damaged DNA templates in mammalian cells, several authors have drawn conclusions that are consistent with the previous model. By means of a temperature-sensitive SV40 mutant (*tsA58*), Sarasin and Hanawalt were able to synchronize viral DNA molecule populations and proposed that viral replication forks are blocked by the first dimer encountered during the first 30 min after UV irradiation and then synthesis past dimers may occur (28). On the other hand, using low UV doses (producing only one to two dimers per viral DNA molecule), it has been proposed that lesions are rapidly bypassed by replication forks (32, 33) and the inhibition of DNA replication is not due to a block of growing fork by dimers but to the nonfilling of gaps left on the replicated molecules (32). More recently, Berger and Edenberg (3) have analyzed, by electron microscopy, viral replicative intermediates (RIs) carrying 5 to 7.5 dimers per SV40 genome and, by mapping the position of replication forks compared with the origin of replication after *Bgl*I restriction endonuclease digestion, they have concluded that dimers block viral growing forks. No evidence has been provided to distinguish whether these blocks were due to dimers located on the template for the leading or lagging strands, and no gaps were detected on the replicating molecules (3).

To study the mechanisms of the block of viral replication forks, we have analyzed the properties of viral RI molecules obtained from infected cells after UV irradiations producing different average numbers of dimers per SV40 molecule. Our previous experiments have shown that the structure of SV40 RI molecules carrying 5.5 dimers is different from that observed for the normal Cairns structure of unirradiated RIs, since they are composed of a circle with a tail resulting from a break at one of two replication forks blocked by a UV lesion (22). We extended our previous studies by analyzing viral RI molecules isolated in UV-irradiated repair-proficient monkey and human cells or in excision repair-deficient xeroderma pigmentosum (XP) human fibroblasts. Electron microscopic analysis of these molecules indicated that replication forks are blocked by UV-induced lesions and may be a substrate for cellular nucleolytic activity, probably related to repair of the blocking lesions, giving rise to the tailed circlelike structure of UV-irradiated RIs. Furthermore, single-stranded DNA portions and sites recognized by the T4 gene 32 single-stranded DNA-binding protein were visualized in UV-irradiated RI molecules. The presence of such portions in these molecules was confirmed by biochemical analysis of the products of digestion with single-strand-specific S1 nuclease. These portions may correspond to gaps opposite the lesions in the lagging strand.

MATERIALS AND METHODS

Cell cultures and virus. Established African green monkey kidney cells (CV1P) were grown in plastic dishes (Falcon) in Dulbecco modified Eagle medium (GIBCO) containing 10% (vol/vol) fetal calf serum (GIBCO) in a 5% CO₂ incubator at 37°C. Wild-type SV40 (strain VA4554) was obtained from P. Berg (Stanford University), and stock virus was produced after infection of CV1P cells with a multiplicity of infection of 10⁻² plaque-forming units (PFU) per cell, purified according to Estes et al. (13). Normal diploid human fibroblasts (HF19) were from R. Cox (Manchester) and XP cells (complementation group A [XP-A], CRL 1223) were obtained from the American Type Culture Collection.

Radioactive labeling of SV40 DNA and UV irradiation. Confluent monolayers of CV1P cells were infected with

SV40 at a multiplicity of infection of 50 PFU per cell and were incubated in medium containing 2% fetal calf serum for 40 h at 37°C. [¹⁴C]thymidine (TdR) at 0.2 µCi/ml (specific activity, 50 Ci/mmol; Commissariat à l'Énergie Atomique France) was added for 24 h to label parental DNA strands. The medium was then removed, and cell cultures were washed with 10 ml of phosphate-buffered saline. Infected cells (10 petri dishes were infected for each dose) were UV irradiated at 254 nm, using a 30-W germicidal lamp at an incident dose of 1.2 J/m² per second. After UV irradiation with 75, 100, 125, and 175 J/m², 3 ml of medium containing 2% dialyzed serum and 50 µCi of [³H]TdR per ml (specific activity, 56 Ci/mmol) was added per petri dish for 45 min. Parallel unirradiated infected cultures were also ³H-labeled for 45 min. In the neutral sucrose gradient experiments, 5 min of ³H-labeling was used for unirradiated cultures in order to have about the same amount of radioactivity as for the UV-irradiated samples. When human cells were used, 35 petri dishes of confluent cultures of HF19 or XP-A fibroblasts were infected with SV40 at the same multiplicity of infection as for monkey cells and were UV irradiated 48 h postinfection with 125 J/m² or were left untreated.

Purification of SV40 RI molecules. Viral DNA molecules were selectively extracted from infected cells by the Hirt procedure (19) whereby NaCl was replaced with CsCl. Supernatants were deproteinized by incubation with 200 µg of proteinase K (Merck) per ml at 37°C for 2 h. DNA samples were then made to a density of 1.7 g/cm³ with CsCl according to the method of Tapper and DePamphilis (31), and density gradients (5 ml) were produced by centrifugation in a Spinco VTI 80 rotor at 60,000 rpm for 15 h at 15°C. Fractions (0.16 ml) were collected from the bottoms of the tubes, and 10 µl of each fraction was assayed for radioactivity according to the method of Carrier and Setlow (6). Peak fractions were pooled and dialyzed against TE buffer (0.01 M Tris hydrochloride [pH 7.6], 0.001 M EDTA) before being chromatographed through benzoyl-naphthoyl-DEAE (BND)-cellulose (Serva, Heidelberg, Federal Republic of Germany) columns, prepared as previously described (22). Supercoiled form I (FI), circular form II (FII), and linear form III (FIII) SV40 DNA molecules were eluted with 1 M NaCl in TE buffer, whereas double-stranded molecules containing single-stranded regions were eluted by using the same buffer containing 50% formamide. Peak fractions corresponding to RI or FI + FII forms were pooled and then dialyzed against TE buffer before being analyzed by neutral or alkaline sucrose gradients or by electron microscopy.

Endonuclease treatments and determination of pyrimidine dimers. Viral DNA samples were incubated with *Bgl*I, *Eco*RI, *Bam*HI, or *Pst*I restriction endonucleases or with single-stranded S1 endonuclease (5 to 50 U) (New England BioLabs, Beverly, Mass.) for 20 min at 37°C in the presence of the respective specific incubation buffers. The reaction experiments were stopped by adjusting samples to 10 mM EDTA–0.1% sodium dodecyl sulfate, and DNA samples were then dialyzed against TE buffer. T4 endonuclease V (from P. Seawell, Stanford University, Stanford, Calif.) digestion was made in the presence of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–100 mM NaCl for 30 min at 37°C. The average number of pyrimidine dimers was determined by calculating the ratio of the molecular mass of linear SV40 single-stranded DNA (1.72 × 10⁶) to that of T4 endonuclease V-digested ¹⁴C-labeled FI DNA, determined by alkaline sucrose gradients.

Sucrose gradient sedimentations. Neutral or alkaline sucrose gradients (5 to 20%) were carried out according to the

TABLE 1. Specification of UV-irradiated RIs in monkey cells

UV-irradiation dose (J/m ²)	Avg. no. of pyrimidine dimers/SV40 DNA molecule	No. of SV40-sized molecules				Tail size (%) ^a	DNA strand size		Avg interdimer distance/strand	
		Total	Tailed circles	Cairns forms	Broken molecules ^b		MDa ^c	% ^a	MDa	% ^a
0	0	125	0	120	5					
75	3.4	135	112	13	10	59	0.99	58	1.03	60
100	4	71	63	5	3	50	0.86	50	0.86	50
125	5.5	101	93	1	7	41	0.72	42	0.63	37
175	7.8	75	71	0	4	29	0.58	31	0.44	26

^a Percentage relative to size of SV40 genome.^b -O-, H-, or Y-shaped.^c MDa, Megadaltons.

method of Sarasin and Hanawalt (28). Dialyzed SV40 RI fractions were concentrated by sedimentation (31) before being loaded on sucrose gradients. The M_n 's were determined as described previously (26). In our experiments, ¹⁴C-labeled linear FIII DNA was used as an internal marker.

Construction of circular heteroduplex double-stranded DNA molecules containing a single-stranded portion. Circular SV40 DNA molecules carrying a 354-nucleotide single-stranded region were obtained by digestion of purified FI DNA either with *Bgl*I or with *Eco*RI and *Hpa*II restriction endonucleases followed by denaturation and hybridization of resulting linear molecules as previously described (1).

Treatment with T4 gene 32 single-stranded DNA-binding protein. ³H-labeled SV40 RI samples were incubated in the presence of 13 µg of purified T4 phage gene 32 single-stranded DNA-binding protein (J. J. Toulmé, Paris, France) for 25 min at 37°C in TE buffer. At the end of the incubation period, samples were made to 10 mM magnesium acetate–1% glutaraldehyde and incubated for a further 10 min at 37°C. DNA samples were then chromatographed by gel filtration through a Sephadex G50 (Pharmacia) column to remove unbound proteins.

Electron microscopic analysis. RI DNA samples of peak fractions from BND-cellulose columns were spread for electron microscopy with formamide and cytochrome *c* according to the method of Kleinschmidt (20) as modified by Davis et al. (10) on 3-mm collodion-coated grids. The Dubochet spreading procedure (11) was used when proteins bound to DNA had to be observed. SV40 DNA molecules were photographed in a Siemens 1000 electron microscope at 12,000× magnification. Electron micrographs were then amplified three times and printed directly onto Rapidoprint FP1-4 papers (Agfa-Gevaert). Contour lengths were measured by a shadowgraph with a Hewlett Packard-type 9830 A calculator.

RESULTS

UV irradiation blocks completion of SV40 DNA replication. Confluent monkey kidney cells (CV1P) were infected with SV40 (VA4554 strain), and at 40 h postinfection they were UV irradiated with 0, 75, 100, 125, or 175 J/m². These UV-irradiation doses produced an average of 0, 3.4, 4, 5.5, and 7.8 pyrimidine dimers per SV40 genome, as determined by the specific T4 endonuclease V (Table 1). DNA molecules synthesized after UV irradiation were pulse-labeled with [³H]TdR, and viral ³H-labeled molecules were then recovered from Hirt supernatants and purified on CsCl density gradients followed by BND-cellulose chromatography. This procedure allowed us to separate fully replicated (supercoiled FI and relaxed circular FII) viral molecules from RI

forms into two fractions that were eluted, respectively, with 1 M NaCl and 1 M NaCl containing 50% formamide buffers. Figure 1 shows that the amount of ³H-labeled material in fractions from a BND-cellulose column containing fully replicated viral DNA molecules decreased progressively with increasing UV doses (panels B to E) compared with the sample from unirradiated infected cells (panel A). This result shows that conversion of SV40 RIs into fully replicated molecules is inhibited by UV irradiation, and the extent of this inhibition is proportional to the UV dose and, consequently, to the amount of UV lesions on viral DNA molecules. In contrast, the amount of ³H-labeled DNA molecules in the fraction containing RI forms remains relatively constant (Fig. 1A to E), indicating that UV irradiation does not change the pool of viral replicating molecules.

SV40 replication fork is blocked by UV lesions encountered on one template strand. To study the molecular mechanism of the inhibition of SV40 DNA replication by UV irradiation, the structure of viral RI molecules was analyzed by electron microscopy. Due to the fragility of molecules containing single-stranded regions in replication loops (35), we used a purification protocol that avoided treatments in which these molecules could be broken, such as phenol-chloroform treatments, ethidium bromide-CsCl gradients, or ethanol precipitations (31). RI molecules obtained from BND-cellulose chromatography were spread for electron microscopic analysis.

Figure 2 shows that viral RI molecules purified from unirradiated infected cells (125 molecules have been observed) display the classical Cairns structure (panels A through D) of replicating molecules, presenting the unreplicated portion in supercoiled (panels A and B) or relaxed form (panels C and D). Less than 4% of the molecules presented the structure of a double-stranded circle with a tail, such as those seen in Fig. 3, or had an -O-, H, or Y-shaped structure (see below and Table 1), indicating that, using this purification procedure, almost all RI molecules presented an intact replication structure. When this material was submitted to digestion with *Bgl*I restriction endonuclease, which cleaves the SV40 genome once at position 5235 in the SV40 physical map (35 base pairs close to the site of replication origin), and then analyzed by electron microscopy, the Cairns structures were converted into H-shaped structures in which the lengths of the arms were identical (Fig. 2F, $a = a' = b = b'$). After digestion with *Eco*RI restriction endonuclease, which cleaves at position 1782 of the physical map (about 110° with respect to replication origin), RI molecules were also converted into H-shaped forms in which the lengths of the arms was $a = a' \neq b = b'$ (Fig. 2G). When these molecules were digested with *Bam*HI,

FIG. 1. Inhibition of completion of SV40 DNA replication analyzed after BND-cellulose chromatography. Chromatographic profiles of BND-cellulose column elutions of SV40 DNA molecules purified from infected CV1P cells nonirradiated (A) or irradiated with UV doses of 75 (B), 100 (C), 125 (D), or 175 (E) J/m². ³H-labeled viral DNA molecules of peak fractions from CsCl density gradients were applied onto BND-cellulose columns as described in Materials and Methods. Arrows indicate elutions with 1 M NaCl and 1 M NaCl–50% formamide buffers.

which cuts at position 2553 (180° opposite the replication fork), -O-shaped forms were observed in which the lengths of the arms were $c = c'$ and $ab = a'b'$ (Fig. 2H). Therefore, in all cases, the segments corresponding to the replicated portions of the molecules (a , a' , b , b' , ab , and $a'b'$ in Fig. 2F, G, and H, respectively) were symmetrical with respect to the origin of replication for the unirradiated samples.

These observations are consistent with the model of SV40 replication in which two replication forks start from the unique replication origin and proceed bidirectionally at the same rate, as has been previously described (5, 17).

In contrast, almost all viral RI molecules obtained from UV-irradiated infected monkey cells display the structure of a circle equal to the size of SV40 genome and a double-stranded DNA tail as in Fig. 3A through E. After irradiation

with 75, 100, 125, and 175 J/m², this tailed structure was detected for 83, 88, 92, and 95% of all RI molecules analyzed (Table 1). Size measurements of these tails revealed a broad distribution of length, with average values corresponding to 59, 50, 41, and 29% of the full-sized SV40 genome which is inversely proportional to the UV doses (Table 1). With a frequency smaller than 5%, circles presenting two tails with different lengths (Fig. 3F, -O-shaped structures) or Y-shaped structures (with asymmetric arms) can be observed in UV-irradiated RI preparations, which may correspond to broken molecules.

To correlate the structure of UV-irradiated RIs observed by electron microscopy with the inhibition of the completion of viral DNA replication and with the presence of pyrimidine dimers on these molecules, two sets of experiments were carried out. First, the size of ³H-labeled DNA synthesized 45 min after UV irradiation was measured by sedimentation of UV-irradiated RI molecules on alkaline sucrose gradients. Second, using the same procedure, the average number of pyrimidine dimers induced on viral parental strands immediately after these UV doses was determined by measuring the average interdimer distance in ¹⁴C-labeled FI DNA after treatment with the dimer-specific T4 endonuclease V. The results have shown that the sizes of single-stranded DNAs newly synthesized after irradiation of infected cells are similar to those found for the distance between two pyrimidine dimers measured after T4 endonuclease digestion. Moreover, we found that the average length of the tail, measured by electron microscopy (Fig. 3), is similar to the size of newly synthesized DNA after UV irradiation and to the average distance between two pyrimidine dimers in the same DNA strand for each UV-irradiation dose (Table 1).

These results indicate that inhibition of SV40 DNA replication by UV irradiation (Fig. 1) is due to the block of one of the two viral replication forks by the dimer encountered on one parental strand during its progression. A break occurring in a single-stranded region on the parental strand close to a dimer in this blocked fork may lead to formation of the tailed structure typical of UV-irradiated RI molecules, as observed by electron microscopy.

To make sure that the tailed structure of UV-irradiated RIs is the result of a break of one replication fork during bidirectional replication and not to the unidirectional rolling-circle replication mode, which might have been induced after UV irradiation, UV-irradiated RIs obtained from infected cells irradiated with 100 J/m² were digested with *Bgl*I and then analyzed by electron microscopy. In the event rolling-circle replication had occurred, digestion with *Bgl*I would convert tailed circles into Y-shaped forms having two symmetrical arms ($a = a'$) with a length identical to the average lengths of the tails of undigested molecules, e.g., 50% of the full genome length. The result of this analysis showed that digestion of UV-irradiated RI molecules with this restriction endonuclease gives rise to Y-shaped forms that always present two symmetrical arms ($a = a'$) whose average length is reduced to 25% of the size of the full genome (Fig. 3G to J). When the size of newly synthesized DNA strands is measured in an alkaline sucrose gradient after *Bgl*I digestion, it is also reduced to 0.4×10^6 , representing half the size of the undigested DNA (Table 1). Therefore, the tailed structure of UV-irradiated RI molecules is the consequence of a break located at the blocked fork and not to a rolling-circle replication mode.

An excision-repair activity acts on lesions, blocking SV40 replication forks. To determine whether the break occurring on growing forks in UV-irradiated SV40 RIs is the result of

FIG. 2. Electron microscopic analysis of unirradiated SV40 RIs. Viral RI molecules from unirradiated infected CV1P cells purified after BND-cellulose column elutions were spread with the formamide-cytochrome *c* Kleinschmidt procedure as described in Materials and Methods (panels A through E). Arrows indicate single-stranded interruptions in RI molecules (panels B through E), in heteroduplex DNA carrying a gap of 354 nucleotides (panel J), or in ϕ X174 single-stranded circular DNA (panel E, right molecule). The same RI molecules were spread after digestion with *Bgl*I (F), *Eco*RI (G), and *Bam*HI (H) restriction endonucleases. Bar, 0.2 μ m.

nonspecific nicking activities associated with the viral replicon (14, 30) or with the endonucleolytic activity of cellular excision repair enzymes which may act on dimers encountered by replication forks, experiments were carried out using human fibroblasts from XP-A donors. Since these cells are known to be deficient in the excision repair activity of pyrimidine dimers (8), the study of SV40 RI molecules obtained from UV-irradiated human fibroblasts will thus provide further information on the nature of the break of viral replication forks. Human fibroblasts from both normal or XP donors are semipermissive toward the SV40 lytic cycle, and the SV40 replication mode is considered to be identical to that in monkey cells (25). Confluent human normal (HF19) or XP-A fibroblasts were infected with SV40 virus, and at 48 h postinfection cultures were UV irradiated with 125 J/m². After 45 min of [³H]TdR pulse, viral RI molecules were purified and their structures were analyzed by electron microscopy. The results showed (Fig. 4, Table 2) that all viral RI molecules from unirradiated HF19 or XP-A fibroblasts presented the usual Cairns structures (Fig. 4A and B), identical to those observed for RIs from unirradiated CV1P cells (Fig. 2). Viral RI molecules from UV-irradiated fibroblasts presented the structure of a circle with a tail for

97% of the molecules isolated from HF19 fibroblasts (Fig. 4C), similar to the results observed for UV-irradiated RI from CV1P cells (Fig. 3), whereas 95% of the molecules from XP-A cells presented a structure similar to that observed for RI from unirradiated cells (Fig. 4D to G).

When replicated portions of these molecules were measured with respect to replication forks, the average length of these portions corresponded to 42% of the total SV40 genome. This value is identical to that found for the size of newly synthesized DNA strands of RIs from CV1P cells and to the average distance between two pyrimidine dimers on the same parental strand after an identical UV-irradiation dose (125 J/m²; Table 1).

Since the structure of RI molecules from HF19 cells is identical to that of RIs from CV1P cells, we conclude that in these experiments the SV40 replication mode after UV irradiation in human cells is identical to that occurring in monkey cells. Viral growing forks are blocked by the first dimer encountered in one strand during their progression. One fork is broken when viral DNA molecules replicate in repair-proficient cells, while in excision repair-deficient XP-A cells, although forks were also blocked during the 45 min after UV irradiation, no break occurs. This confirms that

FIG. 3. Electron microscopic analysis of UV-irradiated SV40 RIs. Viral RI molecules purified from UV-irradiated infected CV1P cells were spread as described in the legend to Fig. 2 for electron microscopic analysis before (A to F) or after (G to J) digestion with *Bgl*I restriction endonuclease. Arrows indicate single-stranded DNA portions or ϕ X174 single-stranded circular DNA (E, upper molecule). Bar, 0.2 μ m.

the break in replication loops observed in viral RI molecules from UV-irradiated cells is not due to artifactual nicking during purification of the DNA but to the cellular repair enzymes acting on dimers blocking replication forks, which are deficient in XP-A cells.

SV40 UV-irradiated RI molecules contain single-stranded gaps. RI DNA molecules contain one or more single-stranded interruptions in growing forks. These gapped double-stranded DNA molecules are selectively eluted by 1 M NaCl buffer containing 50% formamide on a BND-cellulose column. Indeed, SV40 RI molecules obtained from unirradiated infected cells present such gaps close to replication forks when they are analyzed by electron microscopy (Fig. 2B to E). Using the standard spreading procedure of Kleinschmidt (20), these gaps can be clearly distinguished at the replication forks since they appear thinner than double-stranded DNA compared with single-stranded ϕ X174 DNA spread as an internal single-stranded DNA marker (Fig. 2E and 3E). However, in order to characterize the small gaps on viral RIs observed in our experiments, we created specific gapped molecules by hybridization of SV40 DNA molecules either linearized with *Bgl*I digestion or with *Hpa*II and *Eco*RI double digestion, as previously described (1). The resulting heteroduplex molecule is therefore a double-stranded circle of the full size of the SV40 genome containing a single-stranded portion of 354 nucleotides. When these

molecules were spread by the standard procedure and analyzed by electron microscopy, the single-stranded portion could be clearly distinguished from double-stranded DNA (Fig. 2J).

Gaps are also clearly visible in RI molecules obtained from UV-irradiated infected CV1P (Fig. 3A to F), HF19, and XP-A human cells (Fig. 4A to G). These gaps can be seen not only close but also distal to replication forks and in the circles and tails in UV-irradiated RI molecules isolated from CV1P cells (Fig. 3A to F) or HF19 infected cells (Fig. 4C). In the case of RI molecules from UV-irradiated XP-A infected cells, gaps are clearly visible in the replicated portions of the molecules (Fig. 4D to G). This indicates that gaps could not arise directly from cellular excision repair processes.

Although these gaps are clearly distinguished from double-stranded DNA by this spreading procedure, we wanted to confirm that such thinner portions of molecules correspond to single-stranded DNA. We employed a spreading procedure allowing us to reveal these gaps by using the specific single-stranded DNA-binding T4 gene 32 protein as probe. SV40 RI or UV-irradiated RI molecules from CV1P cells were incubated in the presence or absence of this protein and then spread by a procedure described by Dubochet and colleagues (11). This experiment allows us to reveal short gaps, since the protein binds cooperatively on single-stranded DNA, which therefore appears thicker than double-

FIG. 4. Electron microscopic analysis of SV40 RIs purified from human fibroblasts. Viral RI molecules purified from unirradiated repair-proficient (HF19) (A) or -deficient (XP-A) (B) human fibroblasts were spread as described in the legend to Fig. 2. RI molecules from UV-irradiated (125 J/m^2) HF19 (C) or XP-A (D through G) fibroblasts were spread in the same way. Arrows indicate single-stranded portions. Bar, $0.2 \mu\text{m}$.

stranded DNA. Figure 5 shows that this protein was found only in the portions close to the two replication forks in RI molecules obtained from unirradiated samples (panels E and F), whereas with UV-irradiated RI molecules (obtained from infected cells irradiated with 125 J/m^2) the protein was also bound distal to the fork in the circle and at the extremity of the tail (panels G and H). These thicker regions were never seen in mock-treated DNA from RIs (panels A and B) or UV-irradiated RIs (panels C and D). These observations confirm our previous electron microscopic data and clearly indicate that UV-irradiated RIs contain at least one single-stranded interruption in addition to that expected to be seen close to replication forks. Moreover, the Cairns-like struc-

ture of UV-irradiated RI molecules from XP-A cells makes it possible to locate without any ambiguity the gaps in the replicated portions (Fig. 4D to G).

The presence of gaps in UV-irradiated RIs was further confirmed by digestion with S1 nuclease and analysis on neutral sucrose gradients. The results are presented in Fig. 6. RI molecules obtained from unirradiated infected CV1P cells which contain supercoiled unreplicated portions sediment faster than supercoiled DNA (FI) (Fig. 6A, open symbols). Identical sedimentation properties were displayed by control or UV-irradiated RIs from XP cells (not shown). Since these molecules contain gaps close to replication forks, they sediment at the same position as circular FII DNA after S1 endonuclease digestion (Fig. 6A, solid symbols), as has also been found by others (18). UV-irradiated RI molecules obtained from UV-irradiated infected CV1P or HF19 (not shown) cells, which present a tailed-circle structure and contain a gap in the circle additional to that close to the replication fork (Fig. 3), sediment between circular FII and FI (Fig. 6B, open symbols). When these molecules are treated with S1 endonuclease, they are converted into a bimodal population sedimenting as linear duplex DNAs of various lengths from 0.66×10^6 (1 kilobase pair) to the full length of the SV40 genome (Fig. 6B, solid symbols). These results are consistent with the structure of UV-irradiated RIs observed by electron microscopy and indicate that all molecules contain at least one additional S1-sensitive site other than those close to nonarrested growing forks. These extra sites are located on the replicated portion of the molecule, and they could arise from inhibition of synthesis or completion of Okazaki fragments.

DISCUSSION

Completion of viral DNA replication into covalently closed FI molecules is severely inhibited by UV irradiation in a dose-dependent manner (Fig. 1). This inhibition results from the block of viral replication forks mainly by pyrimidine dimers, according to our (22) and previous results of others (12, 28). Sarasin and Hanawalt (28) showed that viral DNA synthesis may occur past dimers 150 min after irradiation, since an average of one dimer was bypassed in DNA molecules carrying about seven dimers per molecule. Essentially the same results were reported by Clark and Hanawalt (7), and they proposed that molecules containing dimers could be converted into complete FI by DNA strand exchanges. We did not detect significant differences between the sizes of newly synthesized DNA strands and the inter-dimer distances in DNA molecules carrying various average numbers of dimers per molecule; no significant exchange of dimers between parental and daughter strands was detected even after 120 min postirradiation labeling time (not shown).

After biochemical analysis of UV-irradiated RIs by dye-buoyant density or neutral sucrose gradients, Sarasin and Hanawalt (28) observed that the superhelicity and sedimentation properties of these molecules differ from those of normal RIs and suggested that a nick occurred on parental strands, giving rise to a different structure. We confirmed these previous biochemical data on UV-irradiated RIs, and we demonstrated by electron microscopy structural differences between UV-irradiated and control RI molecules when repair-proficient cell lines were used. However, using UV-irradiation doses producing several dimers per molecule and the CV1 cell line, Edenberg and co-workers indicated that the structure of RIs, as analyzed by density gradients or electron microscopy, is similar to that of UV-irradiated RIs

TABLE 2. Specification of UV-irradiated SV40 RIs in human cells

Cell line (dosage) ^a	No. of SV40-sized molecules		
	Total	Tailed circles	Cairns forms
HF19 ^b			
0	27	0	27
125	35	34	1
XP-A ^c			
0	24	0	24
125	41	2	39

^a UV-irradiation dose in J/m^2 .

^b Normal repair-proficient human fibroblasts.

^c Excision repair-deficient XP fibroblasts.

FIG. 5. Analysis of single-stranded DNA portions on SV40 RI molecules. Viral RI DNA molecules from unirradiated (A, B, E, and F) or UV irradiated (125 J/m^2) infected CV1P cells (C, D, G, and H) were spread by the Dubochet procedure before (A to D) or after (E to H) incubation with single-stranded DNA-binding protein (T4 gene 32 product). Arrows indicate protein bound to these portions. Bar, $0.2 \mu\text{m}$.

and that no significant bypass of dimers occurs (3, 12, 29). These different conclusions can all be explained by the different experimental procedures employed, e.g., UV-irradiation doses, pulse-labeling DNA procedures, and protocols to synchronize or purify RIs. For example, Williams and Cleaver (33) and White and Dixon (32), using UV doses producing only one to two dimers per molecule, concluded that these dimers are accommodated after irradiation and that no block of growing forks occurs. Under these conditions, a large amount of the DNA molecules (approximately 37%) do not contain pyrimidine dimers and in 50% of the molecules containing one dimer, it would be located in the template for the lagging strand, which does not necessarily block fork progression. These molecules would behave similarly to unirradiated RIs, that is, they would sediment as Cairns structures in sucrose gradients (28S) and display symmetric replicated arms in H-shaped structures when analyzed by electron microscopy after *Bgl*I digestion (32). Such arms were found to be asymmetric in molecules carrying 5 or 7.5 dimers by other authors (3).

In our experiments, we used UV doses producing 3.5 to 7.8 dimers per SV40 genome, thus almost all DNA molecules contained dimers in template for both leading and lagging strands. We labeled viral DNA with short (45 min) postirradiation pulses without further chasing, allowing the study of early events of DNA replication after UV irradiation rather than late events, such as elongation or termination. Moreover, since our electron microscopic analysis was the main part of these studies, we were very careful with the DNA purification procedure in order to obtain viral RI preparations in which almost all (95%) of the molecules presented intact replication loops (Table 1). Indeed, our biochemical and electron microscopic data on UV-irradiated RIs indicate clearly that the block of growing forks by γ -rays gives rise to a structural change in the structure of RI molecules when they are isolated in repair-proficient monkey or human cells;

FIG. 6. Sedimentation analysis on neutral sucrose gradients of SV40 RI molecules after S1 nuclease digestion. Purified ^3H -labeled viral RI molecules from nonirradiated (A) or UV-irradiated (B) infected CV1P cells were analyzed on neutral sucrose gradients as described in Materials and Methods before ($\circ$) or after ($\bullet$) single-stranded specific S1 nuclease digestion. Arrows indicate the sedimentation position of FI, FII, and linear 4 and 1-kilobase-pair restriction fragments obtained after SV40 DNA digestion with *Pst*I endonuclease. Sedimentation directions were from right to left.

FIG. 7. Scheme for a possible model of replication of UV-irradiated SV40 DNA template. UV irradiation induces lesions on both strands of the SV40 supercoiled FI template (A and B). DNA replication starts from a unique origin and proceeds bidirectionally (C). Dimers located on the template for the leading strands block the replication fork progression (D). DNA repair-related cellular cleavage of dimers occurs in vivo, giving rise to tailed-circle molecules containing gaps in the replicated portions (E). S1 nuclease digestion converts tailed structures into linear molecules (F). kbp, Kilobase pairs.

these structures are unchanged for UV-irradiated RIs isolated from repair-deficient XP cells.

Our observations strongly support the model schematized in Fig. 7, dimers located in the template for the leading strand severely block the replication forks; if they are located in the template for the retrograde lagging strand, they do not necessarily represent a barrier for the growing fork but they may halt the synthesis or the termination of an Okazaki fragment, leaving a gap opposite the lesions. We provide direct evidence that UV-irradiated RI molecules contain gaps by analyzing these molecules after S1 nuclease digestion (Fig. 6) and visualizing these gaps (without or with T4 gene 32 protein used as a single-stranded DNA probe) by electron microscopy, whereby they appear located in the replicated portion of these molecules (Fig. 3 to 5). Such gaps would arise from the inhibition of DNA polymerization of Okazaki fragments by UV lesions, as predicted in the model. If the lesions are in the template for the leading strand, they would block replication fork movement (Fig. 7D). A nick at the region of one of the blocked forks or at its proximity could give rise to a double-strand break and yield a tail-circle structure, as represented in Fig. 7E. This nick could be the result of DNA repair-associated endonuclease activity as revealed by the analysis of UV-irradiated RI molecules displaying normal Cairns structure when they are isolated from XP cells which are defective in such a process. Lesions in the template for the lagging strand do not seem to be a good substrate for such cellular repair enzymes. In fact, since in all UV-irradiated RI molecules at least one lesion is produced on each replicating strand, such cleavage would yield θ - or Y-shaped structures as tail circles. In our observations, such structures correspond to only 7% of all the UV-irradiated RI molecules analyzed and they may arise from breakage during DNA purification (Table 1). Therefore, such cellular repair activity seems to be specific for lesions blocking a growing fork, when present in the template for the leading strand.

The inhibition of synthesis of an Okazaki fragment in the lagging strand seems to give rise to the single-stranded

regions observed in the replicated portions of UV-irradiated RI molecules obtained from repair-proficient monkey and human or repair-deficient XP cells (Fig. 3 to 5). The size of such gaps, measured when they are clearly distinguishable from double-stranded DNA, comprises between 50 and 300 nucleotides. Although the accuracy of such measurements is obviously limited, this size is in good agreement with non-completion of the synthesis of an Okazaki fragment.

The sensitivity of almost all UV-irradiated RI molecules to S1 nuclease digestion also confirms the electron microscopic visualization of gaps and their formation in only one of the replicating strands, a result very similar to that previously found for chromosomal replicating DNA (9, 21). The fraction of digested molecules (carrying 5.5 dimers) sedimenting as short fragments of 1 to 2 kilobase pairs corresponds to newly synthesized DNA between two pyrimidine dimers, while the amount of labeled DNA sedimenting as 4- to 5-kilobase-pair duplex molecules corresponds to that of replicated DNA still linked to nonreplicated template despite S1 digestion. This can be due to the inability of S1 nuclease to cleave all single-stranded regions, such as those located in the replication forks. Therefore, in our experimental conditions, the action of this enzyme is limited by the size of the single-stranded DNA target, as has also been observed by others (18).

At present, it is not yet possible to provide evidence that dimers are located opposite gaps in DNA replicating molecules, since they do not seem to be a good substrate for the dimer-specific T4-encoded or bacterial endonuclease (28, 33). In our experiments, digestion of UV-irradiated RIs carrying 5.5 dimers with T4 endonuclease V does not significantly affect their sedimentation properties in neutral sucrose gradients, although a large fraction of linear and Y-shaped molecules (corresponding to 100 to 150% of the full-size SV40 genome) was observed by electron microscopy (not shown). These molecules could result from the cleavage of pyrimidine dimers located close to either replication forks or single-stranded gaps (Fig. 7D).

We have recently visualized by electron microscopy SV40 replication forks blocked by bulky lesions such as acetylaminofluorene (AAF) adducts, induced by chemically damaged DNA template, using specific anti-Guo-AAF adduct antibodies. These AAF-treated RI molecules presented a tailed-circle structure similar to that observed for UV-irradiated RIs and also contained single-stranded gaps due to the inhibition of DNA synthesis by the AAF adducts (1). The anti-Guo-AAF antibodies were always located at the replication forks, indicating that the forks were blocked by the chemical adducts. Thus it seems that the in vivo mechanisms of DNA replication of molecules damaged by AAF treatment or UV irradiation are similar.

The implications of such model of replication in understanding the mutagenesis of viral progeny observed after infection of monkey cells with UV-irradiated SV40 (4, 27) are still a matter for speculation. The presence of UV lesions on the viral template is responsible for a 10^2 -fold increase of mutation frequency in viral progeny obtained from untreated infected cells (4, 27). It is also still difficult to understand the mechanisms by which tailed-circular DNA molecules would give rise to a viable viral particle. It is likely that such intermediate structures containing single-stranded gaps could be the substrate for recombination enzymes which could accommodate lesions by rearrangements of the viral genome, thus producing a complete intact or mutated SV40 DNA molecule. Alternatively, single-stranded DNA induced by UV lesions in these molecules could be a signal for the

expression of mutagenic cellular functions, as has been indicated for the SOS response in bacteria (2). The enhancement of error-prone DNA polymerizing activity could be responsible therefore for the appearance of mutations in the viral genome. In this case, the spectrum of point mutations observed in viral progeny (4) would be produced in the late gap-filling step of daughter strands. Mutations would therefore be expected to occur preferentially during replication of lagging strands. Appropriate protocols for distinguishing mutations fixed in leading or lagging synthesized daughter strands will thus provide further information on the molecular mechanism of the replication of UV-damaged mammalian chromosomes and consequently on UV-induced mutagenesis.

ACKNOWLEDGMENTS

We thank E. Puvion (Villejuif, France) for the electron microscopic facilities, P. C. Seawell (Stanford University, Stanford, Calif.) for the gift of the T4 endonuclease, and J. J. Toulmé (Paris, France) for providing purified T4 gene 32 protein. We are also grateful to A. Benoit, Y. Florentin, R. Cousin, and E. Pichard for excellent technical assistance. One of us (A.S.) is very grateful to P. C. Hanawalt (Stanford University) with whom some of these experiments were initiated.

C. F. M. Menck is a professor of the State University of Rio de Janeiro and during this work had a postdoctoral fellowship firstly from the CNPq (Brazil) and then from the Association pour la Recherche sur le Cancer (Villejuif, France). This work was supported by grants from ARC, from the Fondation pour la Recherche Médicale (Paris), and by grant B16-163 F from the Commission of the European Communities (Brussels, Belgium).

LITERATURE CITED

- Armier, J., M. Mezzina, M. Leng, R. P. P. Fuchs, and A. Sarasin. 1988. *N*-acetoxy-*N*-2-acetylaminofluorene-induced damage on SV40 DNA: inhibition of DNA replication and visualization of DNA lesions. *Carcinogenesis* 9:789-795.
- Bailone, A., S. Sommer, and R. Devoret. 1985. Mini-F plasmid-induced SOS signal in *Escherichia coli* is RecBC dependent. *Proc. Natl. Acad. Sci. USA* 82:5973-5977.
- Berger, C. A., and H. J. Edenberg. 1986. Pyrimidine dimers block simian virus 40 replication forks. *Mol. Cell. Biol.* 6:3443-3450.
- Bourre, F., and A. Sarasin. 1983. Targeted mutagenesis of SV40 DNA induced by UV light. *Nature (London)* 305:68-70.
- Campbell, J. L. 1986. Eukaryotic DNA replication. *Annu. Rev. Biochem.* 55:733-771.
- Carrier, W. L., and R. B. Setlow. 1971. Paper strip method for assaying gradient fractions containing radioactive macromolecules. *Anal. Biochem.* 43:427-432.
- Clark, J. M., and P. C. Hanawalt. 1984. Replication intermediates in UV-irradiated simian virus 40. *Mutat. Res.* 132:1-14.
- Cleaver, J. E. 1968. Defective repair replication of DNA in xeroderma pigmentosum. *Nature (London)* 218:652-656.
- Cordeiro-Stone, H., R. I. Schumacher, and R. Meneghini. 1979. Structure of the replication fork in ultraviolet light-irradiated human cells. *Biophys. J.* 27:287-300.
- Davis, R. W., M. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *Methods Enzymol.* 21:413-428.
- Dubochet, J., M. Ducommun, M. Zollinger, and E. Kellenberger. 1971. A new preparation method for dark-field electron microscopy of biomacromolecules. *J. Ultrastruct. Res.* 35:147-167.
- Edenberg, H. J. 1983. Inhibition of simian virus 40 DNA replication by ultraviolet light. *Virology* 128:298-309.
- Estes, M. K., E. S. Huang, and J. J. Pagano. 1971. Structural polypeptides of simian virus 40. *J. Virol.* 7:635-641.
- Hamelin, C. and Y. Yaniv. 1980. Nicking-closing enzyme is associated with SV40 DNA in vivo as a sodium dodecyl sulfate-resistant complex. *Nucleic Acids Res.* 7:679-687.
- Hanawalt, P. C., and A. Sarasin. 1986. Cancer-prone hereditary diseases with DNA processing abnormalities. *Trends Genet.* 2:124-129.
- Haseltine, W. A. 1983. Ultraviolet light repair and mutagenesis. *Cell* 33:13-17.
- Hay, R. T., and M. L. DePamphilis. 1982. Initiation of SV40 DNA replication in vivo: location and structure of 5' ends of DNA synthesized in the *ori* region. *Cell* 28:767-779.
- Herman, T. M., M. L. DePamphilis, and P. M. Wassarman. 1979. The structure of chromatin at DNA replication forks. Okazaki fragments released from replicating SV40 chromosomes by single strand specific endonucleases are not in nucleosomes. *Biochemistry* 18:4563-4571.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
- Kleinschmidt, A. K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. *Methods Enzymol.* 128:361-377.
- Meneghini, R. 1976. Gaps in DNA synthesized by ultraviolet light irradiated WI38 human cells. *Biochim. Biophys. Acta* 45:419-427.
- Mezzina, M., A. Gentil, and A. Sarasin. 1981. Simian virus 40 as a probe for studying inducible repair functions in mammalian cells. *J. Supramol. Struct. Cell. Biochem.* 17:121-131.
- Moore, P. D., K. K. Bose, S. D. Rakkin, and B. S. Strauss. 1981. Sites of termination of in vitro DNA synthesis on ultraviolet and *N*-acetylaminofluorene-treated ϕ X174 templates by prokaryotic and eukaryotic DNA polymerases. *Proc. Natl. Acad. Sci. USA* 78:110-114.
- Moore, P. D., and B. S. Strauss. 1979. Sites of inhibition of in vitro DNA synthesis in carcinogen- and UV-treated ϕ X174 DNA. *Nature (London)* 278:664-666.
- Ozer, H. L., M. L. Slater, J. J. Dermody, and M. Mandel. 1981. Replication of simian virus 40 DNA in normal human fibroblasts and in fibroblasts from xeroderma pigmentosum. *J. Virol.* 29:481-489.
- Rupp, W. D., and P. Howard-Flanders. 1968. Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation. *J. Mol. Biol.* 31:291-304.
- Sarasin, A., and A. Benoit. 1986. Enhanced mutagenesis of UV-irradiated simian virus 40 occurs in mitomycin C-treated host cells only at a low multiplicity of infection. *Mol. Cell. Biol.* 6:1102-1107.
- Sarasin, A. R., and P. C. Hanawalt. 1980. Replication of ultraviolet-irradiated simian virus 40 in monkey kidney cells. *J. Mol. Biol.* 138:299-319.
- Scaria, A., and H. J. Edenberg. 1988. Preirradiation of host cell does not alter blockage of simian virus 40 replication forks by pyrimidine dimers. *Mutat. Res.* 193:11-20.
- Stahl, H., P. Droge, and R. Knippers. 1986. DNA helicase activity of SV40 large tumor antigen. *EMBO J.* 5:1939-1944.
- Tapper, D. P., and M. L. DePamphilis. 1980. Preferred DNA sites are involved in the arrest and initiation of DNA synthesis during replication of SV40 DNA. *Cell* 22:97-108.
- White, J. H., and K. Dixon. 1984. Gap filling and not replication fork progression is the rate-limiting step in the replication of UV-damaged simian virus 40 DNA. *Mol. Cell. Biol.* 4:1286-1292.
- Williams, J. I., and J. E. Cleaver. 1978. Perturbations in simian virus 40 DNA synthesis by ultraviolet light. *Mutat. Res.* 52:301-311.
- Witkin, E. M. 1976. Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. *Bacteriol. Rev.* 40:869-907.
- Zannis-Hadjopoulos, M., Persico, M., and R. G. Martin. 1981. The remarkable instability of replication loops provides a general method for the isolation of origins of DNA replication. *Cell* 27:155-163.