SIGNIFICANCE OF DIMERS TO THE SIZE OF NEWLY SYNTHESIZED DNA IN UV-IRRADIATED CHINESE HAMSTER OVARY CELLS

JUDITH M. CLARKSON *and* ROGER R. HEWITT

From the Departments of Physics and Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

ABSTRACT DNA synthesized after UV irradiation is smaller than that in unirradiated cells even when pulse-labeling times are increased to compensate for the overall reduction in the rate of DNA replication. By isolating newly replicated DNA, incubating it with dimer-specific endonuclease from *Micrococcus luteus*, and analyzing it on alkaline sucrose gradients, we have been able to demonstrate that this DNA is synthesized in segments corresponding in size to the interdimer distance on the parental strand. In addition, the same DNA analyzed on neutral gradients shows no reduction in molecular weight as a result of UV irradiation and/or endonuclease digestion. Our data are thus inconsistent with the presence of "gaps" in newly synthesized DNA opposite the dimers on the parental strand. We suggest that if such gaps are produced as a result of delayed synthesis around dimers, they are filled before the growing point reaches the next dimer.

INTRODUCTION

A great variety of agents are known that produce damage in DNA. Under conditions where there is a significant level of cell survival some of these lesions are removed, but many remain in the damaged cells. Much of the work to date has concentrated on the ability of cells to circumvent UV-induced thymine dimers that remain in the DNA; cultured human cells excise up to 50% or more of such dimers (Regan et al., 1968), but rodent cells excise very few (Trosko and Kasschau, 1967; Steward and Humphrey, 1966; Lehmann, 1972 *a*; Meyn et al., 1974). Meyn et al. (1974) have shown that, in CHO cells, DNA replicated after UV irradiation has the same dimer content as unreplicated DNA. Thus, such lesions do not act as absolute blocks to DNA replication, but they appear to have a modifying effect on the size of the DNA segments synthesized after irradiation. Several laboratories have demonstrated that the DNA synthesized after UV irradiation is smaller than that in unirradiated cells (Cleaver and Thomas, 1969; Meyn and Humphrey, 1971; Fujiwara and Kondo, 1972, Lehmann, 1972 *a*; Fujiwara, 1972; Chiu and Rauth, 1972). The reduced size is not merely a reflection of reduced rates of DNA synthesis after exposure, since smaller-sized DNA is also synthesized when pulse-labeling times are increased to compensate for this. For short [^{3}H]TdR pulses it is also possible to show that, after irradiation, DNA is syn-

thesized in segments corresponding in size to the interdimer distance on the template strand (Buhl et al., 1972 *a*; Lehmann, 1972 *b*). In addition, Buhl et al. (1974) and Lehmann and Stevans (1975), using marsupial and chick cell lines, showed that photoreactivation to remove some of the dimers results in an increase in the size of these newly synthesized segments. Evidence such as this has led to the hypothesis that, after UV irradiation, DNA synthesis is interrupted by dimers in the template strand leaving gaps that are filled at some later time (see review by Lehmann, 1974).

Rupp et al. (1971), using density gradient techniques, showed for *Escherichia coli* that gaps in the newly synthesized strands are filled by segments of DNA from the parental strand. Dimers, measured as UV endonuclease (endoDNase)-sensitive sites, have also been shown to be distributed between the parental and daughter strands one generation after irradiation (Ganesan, 1974). Several unsuccessful attempts have been made to demonstrate such recombination in mammalian cells (Lehmann, 1972 *b*; Painter, 1974; Buhl and Regan, 1973). However, there is evidence that stretches of DNA approximately 1,000 nucleotides in length are synthesized *de novo* and result in an increase in the molecular weight of the discontinuous stretches of DNA synthesized after UV-irradiation (Lehmann, 1972 *b*; Buhl et al. 1972 *b*). There is still, however, no direct evidence that (*a*) dimers act as blocks to DNA synthesis, and (*b*) DNA synthesis resumes beyond the dimer leaving a gap in the daughter strand opposite the dimer in the template strand. We have tested two consequences of this hypothesis directly by using a dimer-specific endoDNase isolated from *Micrococcus luteus*. The specificity of this enzyme for producing single-strand incisions in DNA at the site of a UV-induced dimer has been demonstrated several times (Kaplan et al., 1971; Paterson et al., 1973; Wilkins, 1973) and all the data we present are consistent with this. The sedimentation in alkaline sucrose gradients of UV endonuclease-digested DNA would test directly for a correspondence in the interdimer distance (UV endoDNase-sensitive sites) and the size of "new" DNA segments. Once this had been demonstrated, sedimentation in neutral gradients would reveal double-strand breaks if such "gaps" in the newly synthesized strand were opposite the dimers on the parental strand.

MATERIALS AND METHODS

Cell Line and Culture Techniques

CHO cells were maintained as monolayer cultures in McCoy's 5A medium (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) with 20% fetal calf serum (GIBCO) as described by Humphrey et al. (1970). For each data point 10^6 cells were plated into 10-cm petri dishes on the day before the experiment.

Labeling and UV Irradiation

The parental DNA was prelabeled by adding [^{14}C]TdR (sp act 50 mCi/mM) to the petri dishes when the cells were plated so that the final concentration was 0.05 μCi/ml. Irradiation was by means of two 15-W General Electric germicidal lamps (General Electric Co., Cleveland, Ohio) emitting predominantly 254-nm light at a fluence of 0.55 J·m^{-2}. The cells at the edges of the plates, which would have been shielded from irradiation by the sides of the dish, were removed with a sterile cotton swab and the remaining cells were washed twice with saline to re-

move traces of medium. After irradiation the cells were incubated in fresh medium for 1 h. At this time [^{3}H]TdR (sp act 50–60 Ci/mM) was added to give a final concentration of 50 μCi/ml in order to pulse label newly synthesized DNA.

In order to isolate the ^{14}C-labeled DNA that had replicated after irradiation, medium containing 10^{-5} M BUdR and 10^{-6} M FUdR was added. 30 min later [^{3}H]TdR was added at a final concentration of 20 μCi/ml for 15 min. This protocol ensured that the size of the ^{3}H-labeled DNA strand was a true reflection of that synthesized after UV irradiation, since immediate addition of the [^{3}H]TdR would result in its addition to incomplete strands synthesized before the irradiation.

Isolation of DNA

The DNA isolation procedure used throughout yielded DNA with a molecular weight of at least 4×10^7 (double stranded). This high molecular weight occurs as a consequence of the removal of the cytoplasm and its associated lysosomes before lysis (D. Vizard personal communication). After trypsinization, cells were pelleted and resuspended in a buffer containing 0.06 M Tris, 0.025 M KCl, 0.01 M EDTA, 0.001 M spermidine, pH 7.2, to which 0.25 M sucrose and 0.1% Triton X-100 were added. The cells were then homogenized with 10–15 strokes in a Teflon pestle hand homogenizer and the nuclei pelleted at 5,000 *g* for 5 min. All procedures were carried out at 4°C. The nuclei were resuspended in SSC (0.15 M NaCl, 0.015 sodium citrate), and incubated for 15 min at 60°C in 10 μg/ml RNase. They were lysed with sodium lauryl sulfate and incubated for 30 min at 37°C in 100 μg/ml pronase. An equal volume of chloroform-isoamyl alcohol (24:1) was added to deproteinize the sample and 2 vol 95% ethanol at −20°C were added to the aqueous layer to precipitate DNA. The DNA pellet was dried and dissolved in 0.002 M Tris, 10^{-3} M EDTA, pH 7.5, resulting in a DNA concentration of approximately 10 μg/ml.

In order to extract the DNA that had replicated in the presence of BUdR after UV irradiation, the nuclei were isolated as described above and lysed with 1% sodium dodecyl sarcosinate (Geigy, Ardsley, N.Y.). Samples were then poured into centrifuge tubes and CsCl was added to a final concentration of 56.3%; the samples were centrifuged in a Beckman 50 Ti angle rotor (Beckman Instruments, Spinco Div., Palo Alto, Calif.) at 37,000 rpm for 60 h at 21°C, and the gradients were fractionated from the top by using an Auto-Densiflow (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N.J.) and a peristaltic pump. Aliquots were removed from each fraction for radioactivity determinations and the fractions containing the hybrid-density DNA were pooled and then precipitated with ethanol. A equal volume of water plus 4 vol ethanol were used to prevent precipitation of the CsCl. These fractions contained more than 90% of ^{3}H-radioactivity and approximately 10% of the ^{14}C-radioactivity.

Endonuclease Assay

An endoDNase activity directly against UV-irradiated DNA was isolated from *M. luteus* by chromatography on DEAE cellulose and DNA agarose. The preparation is devoid of activity against unirradiated DNA in the presence or absence of bivalent cations. We appreciate the cooperation and advice of Dr. Herbert Heijneker, University of Leiden, The Netherlands, in whose laboratory the activity was prepared.

Aliquots of 0.5 ml of the DNA solution were used for the assay. A concentrated solution of the buffer was added to give 0.02 M Tris (pH 7.5), 0.1 M NaCl, 10^{-3} M EDTA, 5×10^{-3} M mercaptoethanol. The samples were all incubated for 30 min at 30°C ± 10 μl of endonuclease.

Sucrose Gradients

Exponential sucrose gradients (Noll, 1967) were prepared from solutions of 5% and 27% sucrose which contained 1 M NaCl and 0.01 M EDTA, and included 0.1 M NaOH for alkaline gradients

TABLE I

MOLECULAR WEIGHT OF DNA AS FUNCTIONS OF UV FLUENCE AND THE AMOUNT AND TIME OF SUBSEQUENT TREATMENT WITH UV ENDODNASE

Dose of UV light	Amount of endonuclease	Incubation time	
		30 min	2 h
	μl		
Control (no UV)	10	28.6×10^6	
$5\ J \cdot m^{-2}$	10	13.2×10^6	12.8×10^6
$10\ J \cdot m^{-2}$	10		8.9×10^6
$20\ J \cdot m^{-2}$	10	5.4×10^6	5.9×10^6
$20\ J \cdot m^{-2}$	25		4.9×10^6

(Lett et al., 1970). Sample (0.5 ml) was applied to the top and gradients were centrifuged at 16,500 rpm for 17 h at 20°C. Fractions of 1.4 ml were collected from the top by siphoning through a Buchler Densiflow. Radioactivity was measured after the addition of 5 ml of Aquasol (New England Nuclear, Boston, Mass.) diluted with 80 ml water per liter for neutral samples and 80 ml 3% acetic acid per liter for alkaline samples.

Median molecular weight distributions were utilized in this study to provide a basis for comparison of the size of parental and newly synthesized DNA. The centroid ($\overline{X}$) of each profile was determined by the equation $\overline{X} = \Sigma Xi\,Yi$, where Xi is the fraction of radioactivity found in gradient fraction number Yi. The median molecular weights represented by the centroids were then determined by reference to the sedimentation properties of bacteriophage T_7 DNA, assuming 12.5×10^6 dalton (alkaline) and 25×10^6 dalton (neutral) molecular weights, using the relationship $D_1/D_2 = (M_1/M_2)^{0.4}$ for alkaline gradients (Studier, 1965) and $D_1/D_2 = (M_1/M_2)^{0.38}$ for neutral gradients (Freifelder, 1970).

RESULTS

In Table I the data are shown for DNA extracted from CHO cells immediately after irradiation with 0, 5, 10, or 20 $J \cdot m^{-2}$ UV light, and then incubated with 10 μl or 25 μl of endonuclease for 30 min or 2 h at 30°C. The resulting molecular weights show a clear response to UV exposure and agree well with previously calculated estimates for the interdimer distances at these doses (Buhl et al., 1972 *a*; Rupp and Howard-Flanders, 1968; Lehmann, 1972 *b*). In particular, our data agree very well with Lehmann's estimates of 9.5×10^6 daltons for 11 $J.m^{-2}$ and 5.6×10^6 daltons for 22 $J \cdot m^{-2}$. These results suggest that the endonuclease is recognizing all dimers under all incubation conditions tested. Thus, treatment for 30 min with 10 μl of UV endoDNase was used in all subsequent studies.

Fig. 1 illustrates the results of a similar experiment. However, in this case cells were incubated for 1 h in fresh medium after irradiation, and then [^{3}H]TdR was added for 8 min (control), 12 min (5 $J \cdot m^{-2}$), 24 min (10 $J \cdot m^{-2}$), or 40 min (20 $J \cdot m^{-2}$) before harvesting. An 8-min pulse in unirradiated cells has previously been shown to label a distinct population of molecules with a molecular weight of 2×10^7 (Gautschi et al., 1973), which is assumed to represent the size of "replicons" (Huberman and Riggs, 1968). This is demonstrated in the first panel. The labeling times for the irradiated

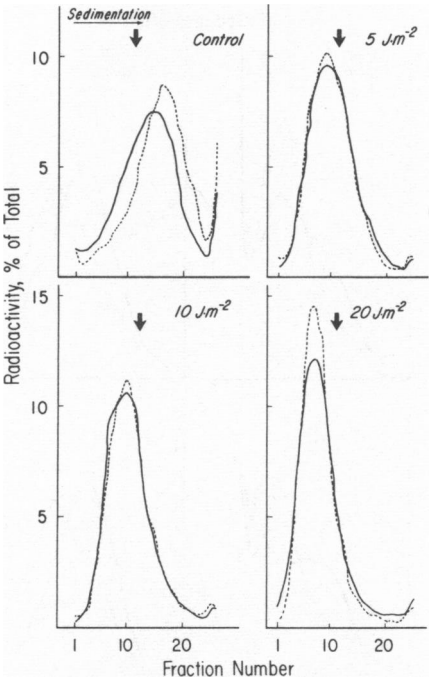

FIGURE 1 Sedimentation profiles in alkaline sucrose gradients of endoDNase-digested DNA as a function of UV fluence. The cells were prelabeled with [^{14}C]TdR, UV irradiated, and incubated in fresh medium for 1 h. [^{3}H]TdR was then added to pulse label the newly synthesized DNA, the duration of the pulse depending on the rate of synthesis: 8 min for control cells; 12 min after 5 J·m^{-2}; 24 min after 10 J·m^{-2}; or 40 min after 20 J·m^{-2}. The cells were harvested and the DNA prepared for endoDNase digestion and analysis on alkaline sucrose gradients. - - - ^{14}C-Prelabeled DNA: —^{3}H-labeled newly synthesized DNA. The arrows mark the position for the sedimentation of T_7 DNA.

samples were increased to compensate for the reduction in the rate of DNA replication caused by each UV fluence as demonstrated by Meyn et al. (1976). It is clear that the increased labeling times do not result in the synthesis of DNA molecules equal in size to those in the control cells. In fact, the profiles exactly correspond to those of the ^{14}C-labeled parental DNA which has been broken at the site of each dimer by the endonuclease.

In Fig. 2 are presented results of our analysis of the effect of UV endoDNase on the double-strand molecular weights of DNA that has replicated after UV irradiation with 5 J·m^{-2}. In order to demonstrate that DNA is replicated in segments corresponding in size to the interdimer distance (as in Fig. 1), short pulses of [^{3}H]TdR (15 min or less) must be used. In this time only 1–2% of the ^{14}C-labeled DNA replicates and the analysis of molecular weight changes in this replicated DNA is obscured by the predominant unreplicated DNA. However, using BUdR in the labeling protocol described in Materials and Methods allows the replicated DNA to be purified, and most unreplicated DNA is thus excluded from analysis.

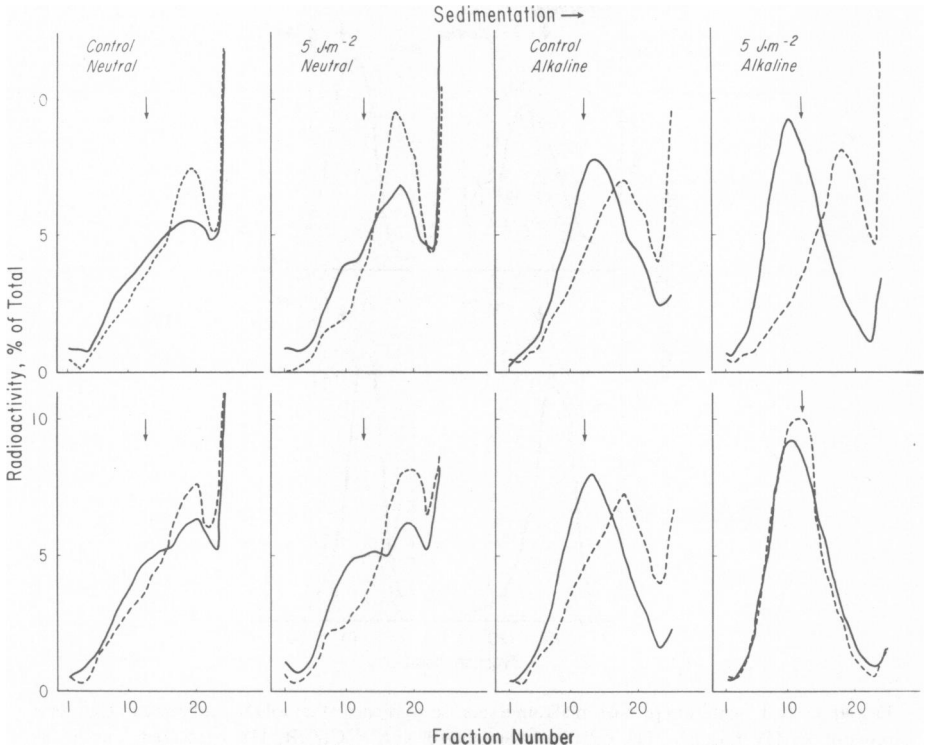

FIGURE 2 The result of endoDNase digestion on the sedimentation profiles of double-stranded and single-stranded newly replicated DNA. Cells were prelabeled with [^{14}C]TdR and UV irradiated with 5 J·m^{-2}. The cells were then incubated in medium containing 10^{-5} M BUdR and 10^{-6} M FUdR and 30 min later [^{3}H]TdR was added for 15 min. The cells were harvested and the DNA that had replicated postirradiation was isolated on CsCl gradients. After ethanol precipitation it was incubated for 30 min with (lower panels) or without (upper panels) UV endoDNase and analysed on sucrose gradients. - - - ^{14}C-Prelabeled newly replicated DNA; —^{3}H-labeled newly synthesized DNA. The arrows mark the position for the sedimentation of T_7 DNA.

The upper panels of Fig. 2 show profiles of this newly replicated DNA in neutral or alkaline sucrose gradients. It is evident that the molecular weight of the double-stranded DNA is the same in the control and the UV-irradiated samples. However, the alkaline gradients show, as in Fig. 1, that UV irradiation with 5 J·m^{-2} results in a reduction in the size of the DNA segments synthesized, in this case resulting in strands with a median molecular weight of 12.3 × 10^6 daltons, as compared with 20.5 × 10^6 daltons in the control sample. The lower panels show the same DNA after treatment with endoDNase. The alkaline gradients show that breaks have been introduced into the parental strand at the sites of the dimers, resulting in a reduction in the molecular weight of the irradiated DNA to 11.9 × 10^6 daltons, a size equal to that of the newly synthesized strands. This value for endoDNase-digested replicated DNA also resembles that in Table I for total parental DNA digested with endoDNase. The same DNA analyzed on neutral gradients shows that the UV endoDNase has little effect on

TABLE II

MOLECULAR WEIGHT OF THE DNA FROM THE NEUTRAL GRADIENTS SHOWN IN FIG. 2

	^{14}C-Labeled replicated parental DNA	^{3}H-Labeled newly synthesized DNA
Control	59.4×10^6	52.7×10^6
Control + endonuclease	65.2×10^6	49.5×10^6
UV (5 J·m^{-2})	58.3×10^6	50.5×10^6
UV + endonuclease	56.0×10^6	44.2×10^6

the molecular weight of either the control or the UV-irradiated DNA. Double-strand breaks at the site of dimers should result in DNA of molecular weight 25×10^6 daltons sedimenting in the same region of the gradient as the T_7 phage DNA. There is little evidence for a peak of DNA at this point. In fact, there is only slight reduction in the average molecular weight as a result of the UV irradiation and/or the UV endoDNase treatment (see Table II).

DISCUSSION

The experiments reported here were designed to determine whether: (*a*) after UV irradiation, DNA is made in segments equal to the size of the template strand in which breaks had been introduced by a dimer-specific endonuclease; (*b*) "gaps" exist in newly synthesized DNA directly opposite the dimers on the template strand so that double-strand breaks would result from digestion with the UV endoDNase.

The data in Table I and Fig. 1 are consistent with previously calculated values for dimer frequency (Rupp and Howard-Flanders, 1968; Buhl et al. 1972 *a*; Lehmann, 1972 *b*) and suggest that the UV endoDNase is recognizing all of the dimers present in the parental DNA strands. In addition, Fig. 1 demonstrates directly that short [^{3}H]TdR pulses predominately label DNA segments equal in size to the interdimer distance, confirming data of Buhl et al. (1972 *a*) and Lehmann (1972 *b*). This suggests two possibilities: (*a*) that DNA synthesis is interrupted at the site of the dimer and reinitiates beyond the "block" leaving a gap; or (*b*) that synthesis proceeds past the dimers, but is slowed to such an extent that there is an apparent accumulation of molecules corresponding to the distance between such obstacles. The first possibility has been extensively expounded (see review by Lehmann, 1974). Lehmann (1972 *b*) and Buhl et al. (1972 *b*) have suggested that gaps of the order of 1,000 nucleotides are left opposite the dimers and are later filled by *de novo* synthesis. This was deduced by a technique that determined the size and frequency of photolabile BUdR-substituted regions in cells pulse-labeled with [^{3}H]TdR postirradiation and then transferred to BUdR medium for several hours.

If such gaps exist opposite dimers it should be possible to demonstrate their existence as double-strand breaks in DNA digested with UV endoDNase. The data presented in Fig. 2 are inconsistent with this assumption. This result does not appear to be a reflection of a reduced ability of the UV endoDNase to act on dimers in newly replicated

DNA, because our analysis demonstrated the same reduction in molecular weight by UV endoDNase treatment of the total parental DNA (Fig. 1) and newly replicated DNA (Fig. 2). In addition, this endoDNase probably attacks dimers specifically in double-stranded DNA (Kaplan et al., 1971). Thus, one might have expected that UV endoDNase could not act on dimers in newly replicated DNA containing large gaps of 1,000 nucleotides suggested by Lehman (1972 *b*) and Buhl et al. (1972 *b*). Thus, this study does not provide support for the notion that large gaps are present in newly replicated DNA. In fact it suggests such DNA possesses the same double-stranded, UV endoDNase-susceptible structure as unreplicated DNA.

Edenberg (1973) and Painter (1974) have previously disputed the presence of gaps in newly synthesized DNA. Edenberg suggested that synthesis is halted for long periods at the dimers rather than continuing after a short delay. This model also poses a problem. Since dimers are present on both strands of the replicating DNA it is necessary that a block on one strand does not stop the replication of the opposite strand which continues until it, too, reaches a dimer. If this were not the case, segments of newly synthesized DNA would, on average, be equal in size to one-half of the interdimer distance.

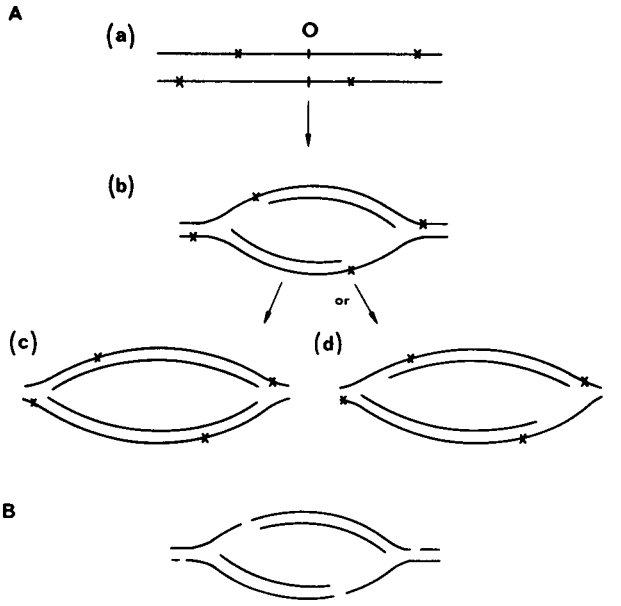

FIGURE 3 The model proposed to resolve some of the discrepancies between our data and previous models. A, The sequence of events in the replication of dimer-containing DNA: (*a*) DNA containing dimers (*x*); 0 represents the point of initiation of DNA synthesis; (*b*) replication has proceeded until a dimer is reached. In the time taken for this to be by-passed, replication proceeds on the opposite strand. The gap is then eliminated (*c*) or moves with the growing fork so that it is no longer opposite the dimer (*d*). B, The result of endoDNase treatment of the structure (*b*). Since only one double strand break occurs on each growing strand, small pieces of DNA will not be released.

The theory we propose below is an attempt to resolve at least some of these discrepancies. It seems likely that DNA synthesis is at least delayed by some UV-induced photoproducts, perhaps dimers. We assume that this accounts for most of the overall reduction in the rate of DNA synthesis and that initiation of replicons is not affected. Thus, at any time after irradiation an increased number of replicons will be operational, resulting in an accumulation of molecules equal in size to the interdimer distance. However, as mentioned in the previous paragraph, a prerequisite for the production of DNA segments of this size is that synthesis continue along the strand opposite the dimer. The delayed synthesis around the dimer would then result in a region of single-stranded DNA between the dimer and the growing fork. DNA synthesis then continues along both strands. In this case the gap can be envisaged as moving with the growing fork, or eliminated by increasing the rate of polymerization on the damaged strand. As illustrated in Fig. 3, even when double-stranded nicks are introduced by the endoDNase, short segments of DNA will not be released. This model predicts that a gap in the vicinity of one dimer must be filled before the next dimer is reached by the growing fork.

This theory is consistent with the recent results of Meneghini (1976) and Meneghini and Hanawalt (1976). Using the single strand-specific endonuclease from *Neurospora crassa*, they demonstrated DNase susceptibility of newly replicated DNA in UV-irradiated WI-38 cells. DNase susceptibility disappeared within 2 h, which presumably represents elimination of gaps in the newly synthesized DNA. However, using the UV specific endoDNase from T_4, they, too, were unable to produce double-strand breaks in newly replicated DNA.

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