STRUCTURE OF THE REPLICATION FORK IN ULTRAVIOLET LIGHT-IRRADIATED HUMAN CELLS

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ABSTRACT The DNA extracted from xeroderma pigmentosum human fibroblasts previously irradiated with 12.5 J/m^2 of UV light and pulse-labeled for 45 min with radioactive and (or) heavy precursors, was used to determine the structural characteristics of the replication fork. Density equilibrium centrifugation experiments showed that a fork moved 6 μm in 45 min and bypassed 3 pyrimidine dimers in both strands. The same length was covered in 15–20 min in control cells. The delay in irradiated cells was apparently due to pyrimidine dimers acting as temporary blocks to the fork movement. Evidence for this interpretation comes from kinetics of incorporation of [^{3}H]thymidine into DNA, which show that the time necessary to attain a new stable level of DNA synthesis in irradiated cells is equivalent to that required for the replication fork to cover the interdimer distance in one strand. On the other hand, the action of S_1 nuclease on DNA synthesized soon after irradiation gives rise to a bimodal distribution in neutral sucrose gradients, one peak corresponding to 43 $\times$ 10^6 daltons and the other to 3 $\times$ 10^6 daltons. These two DNA species are generated by the attack of the S_1 nuclease on single-stranded regions associated with the replication fork. A possible explanation for these results is given by a model according to which there is a delayed bypass of the dimer in the leading strand and the appearance of gaps opposite pyrimidine dimers in the lagging strand, as a direct consequence of the discontinuous mode of DNA replication. In terms of the model, the DNA of 43 $\times$ 10^6 daltons corresponds to the leading strand, linked to the unreplicated branch of the forks, whereas the piece of 3 $\times$ 10^6 daltons is the intergap DNA coming from the lagging strand. Pulse and chase experiments reveal that the low molecular weight DNA grows in a pattern that suggests that more than one gap may be formed per replication fork.

INTRODUCTION

The study of DNA synthesis in damaged templates is of great interest because it may reveal the molecular basis for mutagenesis and carcinogenesis (Witkin, 1969; Caillet-Fauquet et al., 1977). In spite of the extensive investigation devoted to this area, the effect of lesions in the template strands on DNA replication is poorly understood in mammalian cells. The only well-established facts for most mammalian cells are that irradiation with 254 nm light inhibits DNA replication and leads to the synthesis of smaller nascent DNA strands than in unirradiated cells (see review by Lehmann, 1976). Two alternative interpretations for the above facts have been proposed: one (the gap hypothesis) assumes that DNA synthesis proceeds normally in each replication of the irradiated cells, except for a short delay of the replication machinery at the pyrimidine dimers, by far the most abundant lesion introduced in

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DNA by far-UV light. The replication machinery resumes synthesis beyond the dimers, leaving behind gaps opposite the lesions (Lehmann, 1972). Clearly, the delay would explain the inhibition in the overall rate of DNA synthesis and the gaps would explain the shorter nascent strands. According to the other interpretation (the block hypothesis), DNA synthesis is blocked by dimers in each replicon for a longer time; subsequently, synthesis resumes at this very point, without leaving discontinuities (Edenberg, 1976). In this case, the shorter nascent strands would be explained by the accumulation of blocked replicons, because initiation would continue as in control cells.

Recently, we proposed a conciliatory model (Meneghini, 1976; Meneghini and Hanawalt, 1976) that considers two distinct situations (Fig. 1). In the first of these, the dimer is located in the template for the leading (growing in the direction 5′–3′) strand and blocks the fork movement. In the other case, the dimer is located in the template for the lagging (growing in the direction 3′–5′) strand and blocks the termination of a single Okasaki fragment. Due to the discontinuous mode of replication, this block would lead to the formation of a gap. This model

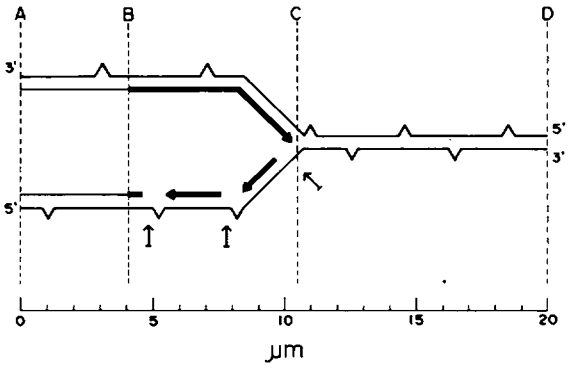

FIGURE 1

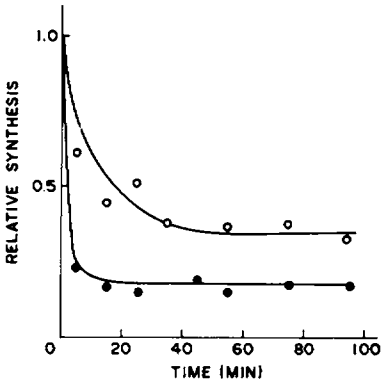

FIGURE 2

FIGURE 1 Effect of pyrimidine dimers on DNA synthesis in mammalian cells. The model assumes that the effect is dependent on whether the dimer is in the template for the leading (5′–3′) strand or in the template for the lagging (3′–5′) strand. In the first case, the dimer will constitute a temporary block to the fork movement. In the second case, the fork will ignore the dimer and progress up to the next origin of an Okazaki fragment. The fragment will be initiated and elongated in a direction contrary to that of the fork movement until the dimer is reached. There, further elongation will be temporarily blocked and a stable gap is formed. The model also incorporates several data obtained in the present work (see text). In particular, it represents the replication forks of XP12RO irradiated with 12.5 J/m^2 of UV light and pulse-labeled for 45 min with radioactive isotope. The molecular weight of these forks, due to shearing during the isolation procedure, is $\simeq 60 \times 10^6$ daltons. _^_, pyrimidine dimers; ———, DNA synthesized before irradiation; ▬▬, DNA synthesized after irradiation incorporating radioactive isotope. The arrows represent single-stranded regions that can be attacked by S_1 nuclease. In the scale at bottom, 1 μm corresponds to 2×10^6 daltons.

FIGURE 2 Relative rate of DNA synthesis at different times after UV radiation. The cells were irradiated with 5.0 (○) or 12.5 (●) J/m^2 and pulse-labeled at various times after irradiation with 10 μCi/ml of [^{3}H]-thymidine for 10 min. Controls without irradiation were run in the same way. The values of specific activity (counts per minute of [^{3}H]-thymidine incorporated into DNA per unit of A_{260}) were calculated as described in Methods and normalized to the control values. The times plotted correspond to the middle points of the pulse-labeling periods after irradiation.

incorporates the crucial predictions of both hypotheses mentioned above, namely, accumulation of forks blocked at dimers and formation of gaps.

Most of the results presented here can be explained by such a model although an alternative model of gaps formed in both strands cannot be excluded. We have employed human fibroblasts derived from patients with the disease xeroderma pigmentosum and transformed by simian virus 40. Since these cells are almost completely defective in excision repair of dimers,[1] interpretations of our results based solely on this phenomenon can be ruled out.

MATERIAL AND METHODS

Cell Culture

Xeroderma pigmentosum (XP) fibroblasts, transformed by simian virus 40 (XP12RO, complementation group A) were routinely grown in glass petri dishes (64 cm^2 surface area) in Dulbecco's modified Eagle's medium, pH 7.0, supplemented with 10% calf serum (Grand Island Biological Co., Grand Island, N.Y.), 472 U/ml penicillin, 94 μg/ml streptomycin and 24 μg/ml ampicillin. The cells were kept in 5% CO_2 humidified atmosphere at 37°C.

UV Irradiation and Cell Labeling

The cells were plated in small glass petri dishes (12 or 20 cm^2) at $\simeq$ ¼ of the confluent density. On the following day, the cells were washed twice with prewarmed buffer solution (PBS) containing 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 1 mM $CaCl_2$, pH 7.0, and irradiated or not with a low pressure germicidal lamp (254 nm, dose rate 0.5 $J/m^2/s$) under a 2-ml layer of PBS. The cells were then supplied with fresh medium containing a radioactive DNA precursor and pulse-labeled for 15–45 min according to the experiment. When chase was required, it was performed in fresh medium containing 10^{-5} M unlabeled thymidine.

Kinetics of DNA Synthesis after UV Exposure

XP cells were plated in 12 cm^2 glass petri dishes and grown until reaching half of the confluent density. They were irradiated with 5.0 or 12.5 J/m^2 in PBS and pulse-labeled for 10 min with 10 μCi/ml of [^{3}H]thymidine (Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N.Y., 60 Ci/mmol) in fresh medium, each plate at a different time after UV treatment. Control plates were treated identically, but not irradiated. Immediately after the pulse, the cells were washed three times with PBS, three times with 5% TCA, and once with 95% ethanol. After air drying, 1.0 ml of 0.3 M NaOH was added to each plate, followed by an overnight incubation at 37°C. From each lysate, 0.3 ml was applied to a piece of Whatman 17 filter paper (Whatman, Inc., Clifton, N.J.), which was oven dried and used to measure the total radioactivity by scintillation counting. Another 0.5-ml sample was diluted twice and used to measure the absorbance at 260 nm. The ratio between radioactivity and absorbance was taken to be a measure of the specific activity of the DNA. These values, compared to those for unirradiated cells gave an estimate of the relative synthesis of DNA after different periods of time after UV treatment.

Elongation of Nascent Strands of DNA

Plates containing XP cells were irradiated with 12.5 J/m^2 and pulse-labeled with 30 μCi/ml of [^{3}H]thymidine (Schwarz/Mann, 60 Ci/mmol) for 45 min. The radioactive medium was replaced by fresh medium containing 10^{-5}M thymidine and the cells were further incubated from 0 to 6 h. In another series of control plates, the unirradiated cells were pulse-labeled for 15 min as above and chased from 0 to 2 h in fresh medium. After the chase period, the cells were incubated with 0.5% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) in saline/EDTA (100 mM NaCl, 10 mM EDTA, pH 8.0) for 2

[1]Menck, C., and R. Meneghini. Unpublished results.

min at room temperature. This treatment released most of the cytoplasm from the cells, and left the nuclei attached to the bottom of the plate. The nuclei were lysed with 0.3 M NaOH, 1 M NaCl, and 10 mM EDTA, either at 37°C for 30 min or at room temperature for 1 h. A sample (200 μl) of each lysate was mixed with 50 μl of an identically prepared cell lysate in which the DNA was labeled with [^{14}C]thymidine (0.2 μCi/ml; New England Nuclear, Boston, Mass., 54 mCi/mmol) for 24 h. Afterwards, the samples were applied to 4.5-ml alkaline sucrose gradients (5–20% sucrose in 0.3 M NaOH, 1 M NaCl, 10 mM EDTA) and centrifuged at 25,000 rpm and 20°C for 120 min in the Spinco SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.). Fractions were pumped out from the bottom of the gradient and dripped onto strips of Whatman 17 paper, which was then washed once in cold 5% trichloroacetic acid (TCA), twice in ethanol, and once in acetone. After drying, the paper strips were cut, placed in vials with 5.0 ml of 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)] benzene (PPO-POPOP)-toluene and their radioactivity measured in a Beckman L-200 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). The ^{3}H counts were appropriately corrected for the spillover of the ^{14}C radiation, with [^{14}C]DNA samples prepared exactly as described above as standards. The relative size of the DNA synthesized after different chase times was calculated by dividing the number average molecular weights (Mn) for the population of [^{3}H]DNA molecules by the Mn for the population of [^{14}C]DNA molecules in the same gradient (see below for calculation of Mn).

Treatment with Single-Strand Specific Endonuclease

After the cells were irradiated, pulse-labeled, and eventually chased, the DNA was extracted as previously described (Meneghini, 1976). The DNA was then incubated with S_1 endonuclease purified from crude α-amylase isolated according to Vogt (1973) up to and including the diethylaminoethyl (DEAE)-cellulose chromatography. The peak fractions exhibiting nuclease activity upon single-stranded DNA were pooled and concentrated by ultrafiltration (Millipore Corp., Bedford, Mass., immersible molecular separator kit; 10,000 = nmwl). Assay solutions contained < 2 μg of DNA in 200 μl of 30 mM sodium acetate, pH 5.0, 300 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol, and 5–60 U/ml of S_1 nuclease (1 U of nuclease activity is the amount of enzyme that solubilizes 1 μg of single-stranded DNA in 1 min at 45°C in 30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, and 5% glycerol; Vogt, 1973). The incubation was performed at 35°C for 30 min, and the reaction was stopped by chilling the samples and adding 50 μl of 0.50 M Tris, 0.05 M EDTA, pH 7.5. Untreated DNA samples were also incubated in the same assay conditions, but without S_1 nuclease. The total sample volume (250 μl) was then applied to 4.0 ml neutral sucrose gradients (5–20% sucrose in 0.1 M NaCl, 10 mM EDTA, pH 8.0) and centrifuged at 32,000 rpm and 20°C for 100 min in the Spinco SW 50.1 rotor. The fractionation and analysis of the gradients were carried out as described above. The molecular weight corresponding to each fraction was determined by the expression of Studier (1965) and λDNA (mol wt, 32.6×10^6) was used as standard. Mn were calculated by the expression $Mn = \Sigma ri/\Sigma(ri/Mi)$ and the weight average molecular weights (Mw) by the expression $Mw = \Sigma(ri \times Mi)/\Sigma ri$, where ri and Mi are the amount of radioactivity and molecular weight of DNA in fraction i, respectively. For Mn calculations the top three fractions were ignored.

Density Labeling and CsCl Gradient Centrifugation

XP cells were irradiated with 12.5 J/m^2 of UV light and then pulse-labeled for 15–45 min with 10^{-5} M bromodeoxyuridine (BrdUrd), 10^{-6} M fluorodeoxyuridine (FdUrd), 10^{-5} M uridine and 10^{-6} M [^{3}H]thymidine (Schwarz/Mann, 50 μCi/ml, 50–60 Ci/mmol) in fresh culture medium. In some experiments, the labeling was preceded by an incubation of the cells for 1 h in medium containing cold BrdUrd, FdUrd, and uridine at the above concentrations (prepulse). The DNA was extracted, treated with S_1 nuclease, and centrifuged in CsCl gradients (7.6 ml.; initial density, 1.72 g/cm^3) at 37,000 rpm and 20°C for 42 h in the Spinco Type 50 Ti rotor. After the upper layer of mineral oil had been carefully removed, the fractions were collected and analyzed as described for the sucrose gradients.

RESULTS

Effect of UV-Irradiation on the Rate of DNA Synthesis and the Size of Nascent DNA

Since the XP12RO cells complementation group A are defective in excision repair, the incorporation of labeled precursors in DNA can be taken as a direct measurement of the overall rate of semiconservative synthesis after UV irradiation. Fig. 2 shows the effect of two different doses of UV light on [^{3}H]thymidine incorporation by XP cells. It can be seen that, after a dose of 5 J/m^2, the rate of DNA synthesis drops gradually, leveling off in 35 min at 35% of the control. These results are very similar to those obtained for Chinese hamster cells (Doniger, 1978). On the other hand, when the UV dose was increased to 12.5 J/m^2, it took only 15 min for the replication rate to reach a stable level of 18% of the control (Fig. 2). Another consequence of the UV treatment was the synthesis of shorter nascent DNA strands by the irradiated cells (Fig. 3). In this and other experiments, the cells treated with different UV doses were also pulse-labeled for different periods of time to attain, in each case, the same

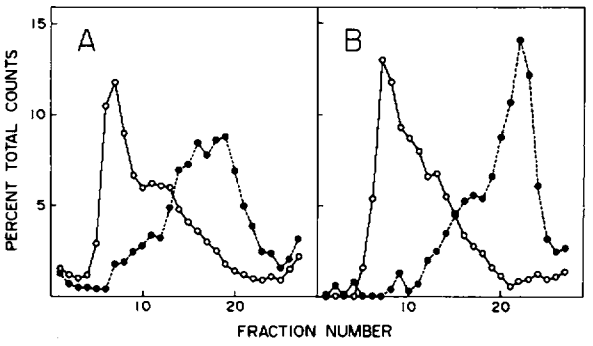

FIGURE 3

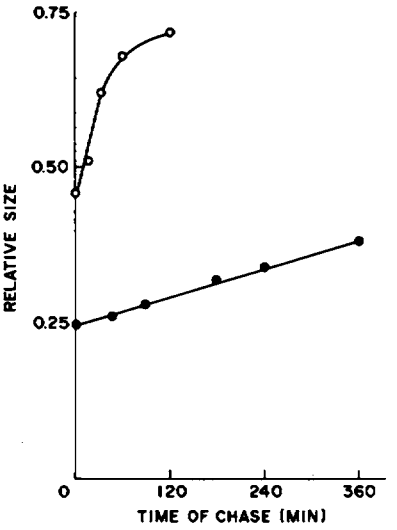

FIGURE 4

FIGURE 3 Size of nascent DNA in irradiated and control cells. The cells were irradiated with 0 (A) and 12.5 (B) J/m^2 and pulse-labeled with 30 μCi/ml of [^{3}H]-thymidine for 15 and 45 min, respectively. The cells were lysed as described in Methods. Identical lysate was prepared from cells that incorporated [^{14}C]-thymidine for 24 h. Samples from the lysates containing [^{3}H]-DNA and [^{14}C]-DNA were applied to alkaline sucrose gradients as described in Methods. Sedimentation is from right to left. 0, [^{14}C]-DNA; •, [^{3}H]-DNA.

FIGURE 4 Growth of nascent DNA. The cells were irradiated with 0 (0) and 12.5 (•) J/m^2 of UV light and pulse-labeled with [^{3}H]-thymidine (30 μCi/ml) for 15 and 45 min, respectively. The medium was replaced by a fresh one containing 10^{-5}M thymidine, and after various times the cells were lysed and sedimented in alkaline sucrose gradients as described in Methods. A sample of a cell lysate containing uniformly labeled [^{14}C]-DNA was also applied to each gradient. The Mn values were determined for the [^{14}C]-DNA and the [^{3}H]-DNA in each gradient. The ratio Mn ([^{3}H]-DNA):Mn([^{14}C]-DNA) (relative size) was plotted vs. chase time in radioactive-free medium.

final amount of DNA synthesized during the pulses. If the labeling periods were the same, the differences in size of the nascent strands could be attributed to slowdown in the rate of DNA synthesis in irradiated cells. In comparison to a 15-min pulse of the control cells, it is necessary to label irradiated cells for 45 min after a dose of 12.5 J/m^2 to obtain the same incorporation level. Although this is in apparent conflict with the data of Fig. 2, where we showed that cells irradiated with 12.5 J/m^2 synthesize DNA at ⅕ of the rate of control cells, it must be pointed out that in the experiment depicted in Fig. 3 the pulse began immediately after UV irradiation and that the rate of DNA synthesis reached its lowest level in ≃ 15 min. It can be seen in Fig. 3 that, even after equal amounts of DNA synthesis, the Mn of nascent strands of irradiated cells (16.5×10^6) is lower than that of control cells (34.8×10^6). The sedimentation profile of nascent DNA strands from irradiated cells displays a peak of 12.5×10^6. As discussed above, these results can be equally explained by the block hypothesis and the gap hypothesis.

Growth of Nascent DNA Strands

The rate at which the initially short nascent strands grow may reveal some characteristics of the defective structures of replication in irradiated cells. We followed this growth through a pulse and chase experiment and the data are shown in Fig. 4. In both control and irradiated cells, the increase in Mn of the nascent strands follows linear kinetics during the first hour. Essentially the same result is obtained if we use Mw instead of Mn to calculate the relative size (results not shown). This information is relevant because for briefly pulse-labeled DNA a calculation of Mw from ^{3}H radioactivity will give a value that corresponds approximately to the Mn for the DNA molecules (Ehmann and Lett, 1973). It can be seen that the growth of the nascent strands is severely impaired in irradiated cells. From these results nothing can be concluded with respect to the rate of the fork movement. This may in fact be blocked by the dimers (Edenberg, 1976) or proceed normally but leaving gaps opposite the lesions (Doniger, 1978). Only information obtained from other types of experiments makes it possible to distinguish between these two possibilities.

Sites Sensitive to S_1 Nuclease in Replicative Structures

Recently we have shown that replicative structures of irradiated human WI-38 cells are susceptible to single-strand specific nuclease from *Neurospora crassa* (Meneghini, 1976). To gain more insight into the features of the replicative structures in irradiated and control cells we have studied this phenomenon more extensively. This time, we used single-strand specific S_1 nuclease with the purpose of attacking single-stranded regions opposite to putative gaps in the replicative DNA. We first verified that, under the incubation conditions used here, concentrations as high as 20 U/ml of S_1 nuclease have no detectable effect on the sedimentation profile of native uniformly labeled DNA, irradiated with 12.5 J/m^2 of UV light (results not shown). This means that pyrimidine dimers are not the sites responsible for the introduction of double-strand breaks in DNA by the enzyme. Fig. 5 depicts the action of S_1 nuclease on pulse-labeled DNA. Again, the pulse-labeling time was such as to result in the same final amount of incorporation in control and irradiated cells. To guarantee that the action of S_1 nuclease on dimers was not contributing to any differential result, the control cells were irradiated at the end of the pulse-labeling period. It should be noted that the DNA from control cells underwent a slight change in Mn upon enzyme treatment, dropping from 55.4×10^6 to 49.5×10^6 daltons. In contrast, the DNA from irradiated cells yielded a bimodal

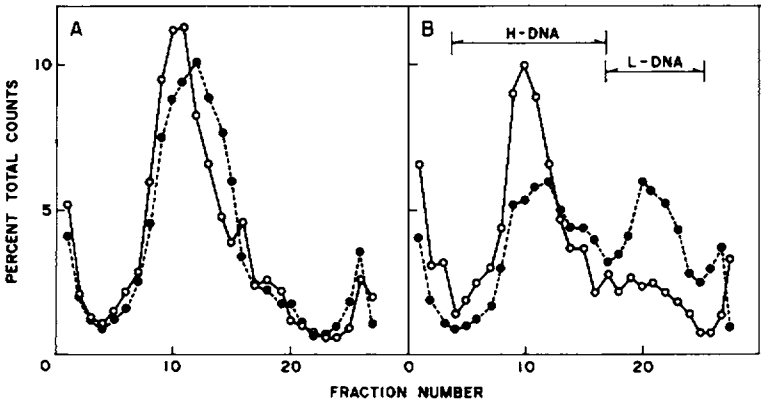

FIGURE 5 Action of S_1 nuclease on nascent double-stranded DNA. In A, the cells were pulse-labeled for 20 min (28 μCi/ml of [^{3}H]-thymidine) and irradiated with 12.5 J/m^2. In B, the cells were irradiated with 12.5 J/m^2 and pulse-labeled with [^{3}H]-thymidine for 45 min. The DNA was extracted and incubated with (●) or without (○) 20 U/ml of S_1 nuclease for 30 min at 35°C. The DNA was then centrifuged in neutral sucrose gradients.

distribution, the Mn of the two new DNA populations (H-DNA and L-DNA) being smaller than that of the untreated DNA. These values are shown in Table I, which includes the results of several experiments similar to the one of Fig. 5, in which either the enzyme activity or the incubation time was varied. This variation affected neither the Mn values nor the percentages of H-DNA and L-DNA. To follow the fate of the L-DNA, we carried out the pulse and chase experiments shown in Fig. 6. The values plotted here represent the percent of counts in the S_1-treated DNA profiles minus the percent of counts in the untreated DNA profiles, for that region of the gradient where the new molecules generated by the enzyme treatment appear (L-DNA). It is clear that the disappearance of the L-DNA is accompanied by an increase in its molecular weight, until it finally merges with the population of H-DNA molecules (not explicitly shown). After 6 h of chase, the L-DNA is only barely detectable and the sedimentation profiles of S_1-treated and untreated DNAs are practically coincident.

Centrifugation of DNA Pulse-Labeled with BrdUrd in CsCl Density Gradient

This technique can provide two types of information: (a) the recognition, under specific conditions, of replicative structures, i.e., the DNA associated with the fork that has a density

TABLE I
MN OF DNA AFTER TREATMENT WITH S_1 NUCLEASE

DNA	Mn × 10^6	Total DNA %	No. of determination
H-DNA (untreated)	57.4 ± 3.9	100	2
H-DNA (treated)	43.1 ± 5.0	71.9 ± 6.6	10
L-DNA (treated)	2.8 ± 0.5	28.1 ± 6.5	10

Data are from several experiments analogous to that of Fig. 5, but varying the enzyme concentration (5–60 U/ml) or incubation time (5–60 min). The values of Mn for peaks in the regions H and L, indicated in Fig. 5B, were calculated as indicated in Methods.

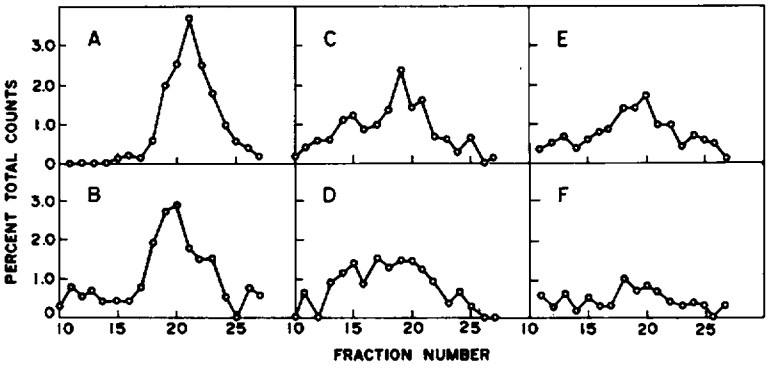

FIGURE 6 Fate of the L-DNA. The cells were irradiated with 12.5 J/m^2 of UV light, pulse-labeled for 45 min with 30 μCi/ml of [^{3}H]-thymidine and chased for various times in radioactive-free medium. The DNA was extracted, treated with 20 U/ml of S_1 nuclease for 30 min at 35°C and sedimented in neutral sucrose gradients. The values plotted are the differences between the percent total counts of the sedimentation profiles of treated and untreated DNA, in the region of the gradients where new molecules produced by the enzyme treatment appear. Chase times and percent of total counts are: (A) 0 min, 21.4%; (B) 45 min, 23.5%; (C) 90 min, 21.1%; (D) 180 min, 19.4%; (E) 240 min, 17.2%; (F) 360 min, 9.9%.

intermediate between the nonsubstituted and hybrid DNAs (Kato and Strauss, 1974; Fujiwara and Tatsumi, 1976); (*b*) the overall rate of fork displacement, which is reflected by the density distribution profile of the DNA labeled with BrdUrd (Painter and Schaeffer, 1969; Cordeiro and Meneghini, 1973). Therefore, this technique permits one to ascertain whether replicative structures accumulate in irradiated cells, as predicted by the block hypothesis. Fig. 7 shows an experiment in which the cells were prelabeled with cold BrdUrd, irradiated with 0 (Fig. 7A) or 12.5 (Fig. 7B) J/m^2 of UV light, and then pulse-labeled with a mixture of 10^{-5} M BrdUrd and 10^{-6} M [^{3}H]thymidine for 20 and 45 min, respectively. This mixture has practically the same effect as that of pure [^{3}H]BrdUrd. The prepulse with cold BrdUrd prevents end-to-end association of thymidine and BrdUrd containing DNAs and

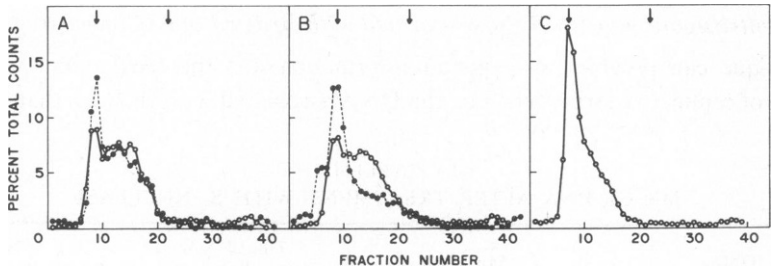

FIGURE 7 Density gradient centrifugation of DNA pulse-labeled with BrdUrd. The cells were incubated in medium containing 10^{-5}M BrdUrd, 10^{-6}M FdUrd, and 10^{-5}M uridine for 1 h. After irradiation with 0 (A,C) or 12.5 (B) J/m^2 of UV light, the cells were pulse-labeled with a medium containing 10^{-5}M BrdUrd, 10^{-6}M FdUrd, 10^{-5}M uridine, and 50 μCi/ml of [^{3}H]-thymidine (10^{-6}M) for 20 min (A) or 45 min (B,C). The DNA was extracted, incubated with (●) or without (0) 60 U/ml of S_1 nuclease for 30 min at 35°C, and centrifuged in neutral CsCl gradient. Arrows at left and right correspond to the densities of hybrid and normal DNA, respectively.

therefore rules out this interpretation as the source of the DNA of density intermediate between the hybrid DNA and the normal density DNA (indicated by arrows in Fig. 7). This procedure would label the AB daughter strand segment of Fig. 1 with cold BrdUrd and the BC daughter strand segment with BrdUrd/[^{3}H]thymidine. The most plausible interpretation for the intermediate DNA of Fig. 7A and B is that it corresponds to replication forks, in which two branches are newly-replicated DNA (and thus contain BrdUrd) and the third is normal density DNA still to be replicated. Another criterion used to characterize the intermediate DNA as being associated with replication forks is its transient character. In fact, this DNA is transformed in hybrid DNA if (*a*) the cells are incubated in cold BrdUrd after the pulse with BrdUrd/^{3}H]thymidine (experiment not shown), and (*b*) the DNA is treated with S_1 nuclease before centrifugation. In this case, the digestion by the nuclease of the single-stranded region of the fork (Fig. 1) would release one of the replicated branches, corresponding to totally hybrid DNA. This is distinctly observed in the case of irradiated cells (Fig. 7B). It should also be noted in the same figure that $\simeq$ 50% of the intermediate DNA remains as such upon the S_1 treatment. This is not due to a lack of enzyme and (or) to short incubation times since the conditions employed in the experiment of Fig. 7 correspond to maximum activity as shown in Table 1. Therefore, the intermediate DNA still remaining after S_1 nuclease treatment in Fig. 7B should correspond to the second replicated branch of the fork, still connected to the unreplicated DNA. In Fig. 7A it can be clearly seen that the intermediate DNA from unirradiated control cells is refractory to density displacement caused by S_1 nuclease. Although some single-stranded regions are expected in replication fork structure, it seems that there is a high predominance of all-duplex forks in eukaryotic cells (Kriegstein and Hogness, 1974), thus explaining this result. It is important to note in Fig. 7A-B that the profiles of the untreated DNAs from irradiated and control cells are practically the same under conditions of equal amount of incorporation of the heavy precursor. Because both DNA preparations have the same Mn (55×10^6) this means that they were labeled with BrdUrd to the same extent in their double strands. Therefore, these results do not support the hypothesis of accumulation of blocked replicons in irradiated cells. Fig. 7C shows the profile of the DNA from control cells pulse-labeled with Brd Urd/[^{3}H]thymidine for the same time as irradiated cells (45 min); it is evident that most of the intermediate DNA has been transformed into hybrid DNA as a result of further elongation. This process is delayed in irradiated cells, probably due to a temporary halt of the fork at the lesions (Fig. 7B). Therefore, initiation of new replicons does not seem to contribute preponderantly to the final amount of DNA synthesized in irradiated XP12RO cells; instead, the overall process of chain growth is delayed, probably during the bypass of the dimers in the leading strand.

Let us suppose that the L-DNA corresponds to the intergap regions of DNA released by the action of S_1 nuclease on gaps. If this is correct, irradiated cells pulse-labeled with [^{3}H]BrdUrd should, upon S_1 treatment, give rise to L-DNA hybrid density. To test this hypothesis we employed an experimental design similar to the one used in the experiment of Fig. 7, except that the cells were not labeled with cold BrdUrd before the irradiation. In this way, we may be sure that any hydrid DNA formed upon S_1 nuclease treatment comes from regions synthesized after UV-irradiation, as we expected for the L-DNA. When the DNAs from control and irradiated cells were centrifuged in CsCl density gradients the result obtained were those shown in Fig. 8 A and B. In contrast to the experiment in which the cells

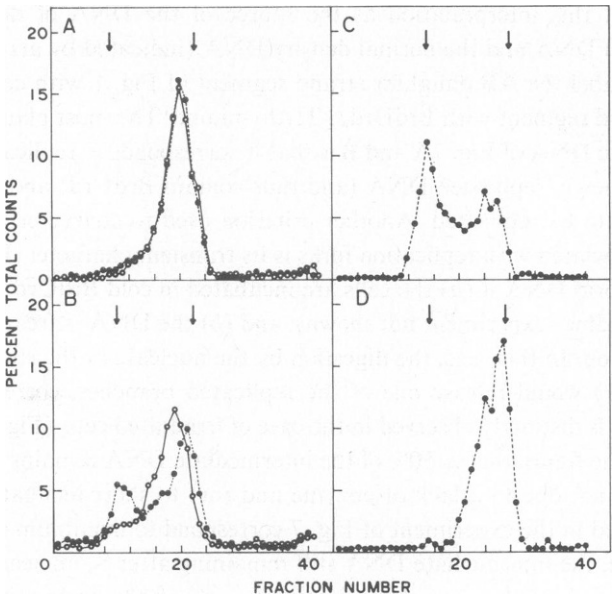

FIGURE 8 Resolution of L-DNA by density centrifugation. The cells were irradiated with 0 (A) of 12.5 (B, C, D) J/m^2 of UV light and incubated with medium containing 10^{-5}M BrdUrd, 10^{-6}M FdUrd, 10^{-5}M uridine, and 50 μCi/ml of [^{3}H]-thymidine for 20 min (A) or 45 min (B, C, D). The DNA was extracted and incubated with (●) or without (○) S_1 nuclease at 35°C for 30 min. In A and B, the DNA was directly centrifuged in CsCl gradient. In C and D the DNA was first sedimented in the neutral sucrose gradient; fractions corresponding to the L-DNA (C) and H-DNA (D) (see Fig. 5B) were pooled, dialyzed, and centrifuged in CsCl gradient. Arrows at left and right correspond to densities of hybrid and normal DNA, respectively.

were prelabeled with cold BrdUrd, no distinct peak of hybrid DNA was observed when no previous treatment with S_1 nuclease was performed. In this case, the intermediate DNA from both the control and irradiated cells is due not only to replicative structures but also to end-to-end associations between normal and BrdUrd containing strands. When these DNAs are previously treated with S_1 nuclease, the profiles of the density gradient centrifugation are quite different for the control and irradiated cells (Fig. 8A and B). In the former case, there is no change in the density distribution of the enzyme-treated DNA molecules (Fig. 8A), whereas in the DNA from irradiated cells a distinct peak of hybrid DNA is formed concomitantly with a slight drop in density of the remaining DNA (Fig. 8B). These are exactly the results that would be expected from the action of S_1 nuclease upon the single-stranded regions of replication forks, pulse-labeled with [^{3}H]BrdUrd, having the structure shown in Fig. 1. If these interpretations are correct, the hybrid DNA from Fig. 8B should correspond exactly to the L-DNA from neutral sucrose gradients. To verify this point, the DNA used in the experiment of Fig. 8B was first treated with S_1 nuclease and then centrifuged in neutral sucrose gradient. The result (not shown) was essentially identical to that depicted in Fig. 5B. The fractions corresponding to L-DNA and H-DNA were dialyzed and centrifuged in CsCl density gradients. Figure 8C shows that most of the L-DNA has the density of hybrid DNA, whereas the H-DNA (Fig. 8D) exhibits only intermediate density.

DISCUSSION

Lesions produced by UV irradiation in the DNA template strands pose obstacles to the replication machinery; as a consequence, abnormal replicative structures are formed. These replicative structures can be conveniently monitored as the intermediate density DNA in density centrifugation of DNA extracted from cells pulse-labeled with BrdUrd (Fig. 7). In the present paper, we have shown that labeling pauses at the replicative structures considerably longer in irradiated than in control cells (Fig. 7). The most plausible interpretation is that dimers delay the fork displacement. This is also suggested by the time elapsed between the irradiation of the cells and the leveling off of the new rate of DNA synthesis (Fig. 2). This time corresponds to 35 and 15 min when the cells are irradiated with 5.0 and 12.5 J/m^2, respectively. These are approximately the times required for the replication fork to cover the interdimer distances. Therefore, the shape of the kinetic curves might reflect successive halt of replication forks, which were, at the moment of irradiation, at distances from the next dimer varying between zero and the full interdimer space. Nevertheless, this halt of the replication fork movement by dimers is not so severe in these cells that bypass of dimers cannot occur. In fact, in the experiments depicted in Fig. 7A and B and 8A and B, an equivalent density displacement of DNA was observed in control and irradiated cells labeled to give the same amount of incorporation. It seems valid to conclude that, in both cases, the replication fork must have traveled equal distances. The rate of fork movement in different mammalian cells in culture, determined by different techniques, varies in the range 0.3–0.6 μm/min (Painter and Schaefer, 1969; Edenberg and Huberman, 1975; Povirk and Painter, 1976). Assuming for XP12RO the lower value, we conclude that a fork should have moved 6 μm in control cells. Our results (Fig. 7 and 8) suggest that the same distance is covered in irradiated cells in 45 min. Because the interdimer distance for a dose of 12.5 J/m^2 corresponds to 4 μm in XP12RO cells,[1] a given fork must have passed 3 dimers in both strands.

These data were incorporated into the scheme of Fig. 1 in an attempt to envisage an average structure of the replication fork of the irradiated cells obtained via our experimental procedure. For this purpose we also took into account the facts that: (*a*) the molecular weight of the double-stranded DNA falls in the range of 55–60 $\times$ 10^6 daltons (Table I) due to shearing during extraction; (*b*) the replicated branches A-C contain DNA synthesized before (A-B) and after (B-C) irradiation, as indicated by comparison of the profiles of untreated DNA in the experiments of Figs. 7B and 8B. The relative lengths of the portions AB, BC, and CD in Fig. 1 were calculated from the average density displacement in the profiles of Fig. 7B and 8B. Assigning a relative density to each fraction in the gradient on a scale varying linearly from zero (density of normal DNA) to one (density of hybrid DNA), the untreated DNAs of the experiments of Figs. 7B and 8B correspond to average relative densities of 0.70 and 0.38, respectively (see Appendix). Therefore, in Fig. 1 we envisage the replicated branch AC as encompassing 70% of the total structure, with the two segments BC synthesized during the pulse representing 38% of the total structure.

It should be emphasized that the scheme of Fig. 1 incorporates ideas that remain to be proven and hence should be considered as a working hypothesis. In particular we propose that dimers in the template for the leading strand block temporarily the fork movement. We also propose that this would not occur with the dimers at the template for the lagging strand,

which would nevertheless block elongation of the single Okasaki fragment, leading to the formation of gap. In other words, DNA polymerization would be temporarily blocked by dimers in both strands, whereas the movement of the fork would be dependent on concurrent DNA polymerization only in the leading strand. Although speculative, the structure depicted in Fig. 1 is in accordance with the results obtained with S_1 nuclease. As a result of attack at the single-stranded regions (indicated in Fig. 1 by the arrows), this enzyme releases $\simeq$ 30% of the label as a distinct peak (L–DNA) of 3×10^6 daltons (Fig. 5 and Table I). Actually, such attack should give rise to a mixture of fragments of sizes corresponding to the full and the half interdimer distance, with an average Mn of 6×10^6 daltons. However, it should be borne in mind that estimates of Mn in this range of the gradient are very crude and may be in error by up to 50%. The results of Fig. 6, showing that the L-DNA tends to decrease in relative amount and increase in size with time, are most reasonably explained by assuming that bypass of the dimer by the leading strand is a faster process than gap filling. Therefore this latter process would be the rate limiting step in the growth of nascent strands. In this way more than one gap may be formed per fork. If gaps are not necessarily filled in the same order as they are formed, the one closer to the fork might be filled first, resulting in an increase in the Mn of the L-DNA, as shown in Fig. 6. After treatment with S_1 nuclease, 60–70% of the total DNA consists of molecules whose size (Fig. 5B and Table I) and density (Fig. 8B) are slightly lower than those of the original population. Because we observed that over 50% of the DNA migrates at the position of intermediate density even after treatment with S_1 nuclease (Fig. 7B), this H-DNA must carry the unreplicated branch of the fork. Therefore, the H-DNA might correspond to the leading strand, linked to the unreplicated branch (segment AD in Fig. 1), which is cleaved from the lagging strand by the S_1 treatment. This cleavage would explain the drop in density of the major peak shown in Fig. 8B.

Presently an alternative model that predicts the formation of gaps in both the nascent strands of the replication fork cannot be excluded. In this case, the nature of the H-DNA should be ascribed to molecules that contained gaps that were filled in during the period of pulse. Although the pulse and chase experiment of Fig. 6 shows that gaps are filled in rather slowly in these cells, it is conceivable that some gaps could be repaired quickly. Experiments are now under progress to elucidate this point.

In bacteria, gaps seem to be formed in both DNA strands (Rupp and Howard-Flanders, 1968; Ganesan, 1974). This also seems to be the case when XP variants are incubated in the presence of caffein after UV irradiation.[2] At the other extreme in systems like mammalian mitochondrial DNA (Clayton et al., 1974) and simian virus 40 DNA (Sarasin and Hanawalt, 1978), the dimer seems to constitute an absolute block to further DNA replication. Equivalent results were obtained with Hela cells, by DNA fiber autoradiography (Edenberg, 1976) and confirmed by us, through density gradient experiments (Meneghini et al., 1978 *a,b*). These differences, which may reflect subtle features of the replication machinery and (or) chromosome structure, imply that due caution is required when attempting to generalize observations from one single system.

It is not within the scope of the present paper to determine how dimers are circumvented by the replication machinery. However, it is implicit here that the circumvention of the dimer in

[2]Lehmann, A. R. Personal communication.

the leading strand is by some replicative bypass process (Higgins et al., 1976; Fujiwara and Tatsumi, 1976), whereas in the gap filling process a different mechanism may be involved requiring either de novo synthesis (Lehmann, 1972) or recombination (Meneghini and Hanawalt, 1975 and 1976; Meneghini et al., 1978*b*).

We thank Dr. A. R. Lehmann and Dr. F. Quina for critically reviewing the manuscript.

This work was supported by a grant (77/1590) from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), São Paulo, Brazil.

Received for publication 22 December 1978 and in revised form 16 April 1979.

APPENDIX

Calculation of the Average Relative Density of the DNA Molecules

Assume that one has a population of DNA molecules possessing regions of different lengths labeled with BrdUrd. Let us define the relative density of any given molecule as $d_i = \rho - \rho_0/\rho_h$, where ρ is the actual density, ρ_0 is the density of the unsubstituted DNA, and ρ_h is the density of hybrid molecules, which have their full length labeled with BrdUrd. Thus defined, d_i is directly related to the fraction of the length of the molecule that has been labeled with BrdUrd.

Let us further define the average relative density of the population as $d_a = \Sigma n_i d_i / \Sigma n_i$, where n_i is the number of molecules with relative density d_i; d_a varies from 0 to 1 and represents the average fraction of labeling of the molecule by BrdUrd.

Finally, one may reasonably assume that the average molecular weights are essentially equal for the DNA populations of different d_i. If the density label is also the radioactive label, as in the present case, one has that: $n_i = k\, r_i/d_i$, where k is a constant independent of i, and r_i is the total radioactivity contained in the fraction of density i. Simple substitution for n_i in the expression for d_a gives: $d_a = \Sigma r_i / \Sigma r_i/d_i$.

In practice, d_i is readily calculable when the density gradient is linear as in the present case. All values in the range $0 < d_i \leq 1.0$ are considered.

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