EFFECTS OF ULTRAVIOLET IRRADIATION ON THE RATE AND SEQUENCE OF DNA REPLICATION IN SYNCHRONIZED CHINESE HAMSTER CELLS

RAYMOND E. MEYN, ROGER R. HEWITT,
LAURENCE F. THOMSON, *and* RONALD M. HUMPHREY

From the Departments of Physics and Biology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

ABSTRACT The effects of ultraviolet light (UV) irradiation on the rate of DNA replication in synchronized Chinese hamster ovary (CHO) cells were investigated. A technique for measuring semiconservative DNA replication was employed that involved growing the cells in medium containing 5-bromodeoxyuridine and subsequently determining the amount of DNA that acquired hybrid buoyant density in CsCl density gradients. One of the advantages of this technique was that it allowed a characterization of the extent of DNA replication as well as rate after irradiation. It was found that while there was a dose-dependent reduction in the rate of DNA replication following UV-irradiation, doses of up to 10 J/m^2 (which produce many dimers per replicon) did not prevent the ultimate replication of the entire genome. Hence, we conclude that dimers cannot be absolute blocks to DNA replication. In order to account for the total genome replication observed, a mechanism must exist that allows genome replication between dimers. The degree of reduction in the rate of replication by UV was the same whether the cells were irradiated at the G1-S boundary or 1 h into S-phase. Previous work had shown that cells in early S-phase are considerably more sensitive to UV than cells at the G1-S boundary. Experiments specifically designed to test for reiterative replication showed that UV does not induce a second round of DNA replication within the same S-phase.

INTRODUCTION

The effects of ultraviolet light (UV) on deoxyribonucleic acid (DNA) synthesis in cultured mammalian cells have been extensively investigated (Cleaver, 1965; Klimek and Vlasinova, 1966; Djordjevic and Tolmach, 1967; Domon and Rauth, 1968). In many of these studies, an attempt was made to correlate the UV-induced depression of DNA synthesis with the observed changes in cell progression and colony-forming ability following UV-irradiation. Domon and Rauth (1968) showed that mouse L cells irradiated in late G1 or early S-phase undergo a dose-dependent delay in progression to mitosis and that this delay occurs entirely in S-phase. Furthermore, Rauth (1967) found that incubation of UV-irradiated cells in caffeine synergistically reduced colony-forming ability and the kinetics of this action of caffeine have been interpreted as indicating that UV-damaged sites are altered during the passage of the cell through S-phase (Domon and Rauth, 1969). These findings coupled with the demonstrations that

mammalian cells can recover colony-forming ability between fractionated doses of UV (Todd et al., 1969; Humphrey et al., 1970; Domon and Rauth, 1973) have led to the postulation that mammalian cells possess an S-phase specific system for recovery from UV-damage. Although many details of the molecular mechanisms are still obscure, it appears that mammalian cells may bypass UV-induced photoproducts during DNA replication in a manner similar to that proposed for bacterial cells by Rupp and Howard-Flanders (1968). A more complete review of this subject may be found in Cleaver (1974) and Lehmann (1974).

In order to ultimately delineate the mechanism responsible for S-phase recovery, it will be necessary to understand in much greater detail the alterations in semiconservative DNA replication caused by UV-irradiation. As a means to this end, we have recently devised techniques for measuring semi-conservative DNA replication in synchronized Chinese hamster ovary (CHO) cells (Meyn et al., 1973; Meyn et al., 1975). This procedure involves growing cells synchronized to the G1-S phase boundary in medium containing 5-bromodeoxyuridine (BUdR) and subsequently determining the amount of DNA that acquires hybrid buoyant density in CsCl gradients. This method has several advantages over other methods of measuring DNA synthesis. First, it yields information about the rate and extent of DNA replication. Second, it allows a clear distinction to be made between semiconservative DNA replication during progression through S-phase and other types of DNA synthesis such as repair replication.

We have utilized this technique to study the rate and sequence of semi-conservative DNA replication in UV-irradiated CHO cells synchronized at the beginning of S-phase. The results of these experiments show that, while there is a UV-dose dependent reduction in the rate of DNA replication, following doses of up to 15 J/m^2 nearly all of the DNA ultimately replicates. Furthermore, UV-irradiation does not induce a second round of DNA replication within the same S-phase.

MATERIALS AND METHODS

CHO cells were used in all experiments. Stock cultures were maintained in McCoy's 5A medium supplemented with 20% fetal calf serum. Irradiation techniques have been described previously (Humphrey et al., 1963). All incubations were carried out at 37°C in a 5% CO_2 atmosphere. Under these growth conditions, the cells have a generation time of 11.5 h consisting of a 2.5-h G1, 6.75-h S, 1.75-h G2, and 0.5-h M.

Complete details of procedures for cell synchronization and analysis of DNA replication in BUdR have been reported elsewhere (Meyn et al., 1973). Briefly, 10^7 cells were seeded into 32-oz prescription bottles and labeled with [^{14}C]thymidine (0.25 μCi/ml, 33 mCi/mM) plus 2 μg/ml thymidine (TdR) for 18 h. After labeling, the cells were first parasynchronized with 7.5 mM TdR and finally synchronized by the mitotic selection technique. Mitotic cells (95% mitotic index) were then plated into 2 mM hydroxyurea (HU) for 9 h to block cells at the beginning of S-phase. Cells were either irradiated at this time or 1 h after removal of HU.

After irradiation, replication test medium containing 50 μg/ml BUdR and 0.1

μg/ml 5-fluorodeoxyuridine (FUdR) was added to the cultures which were then returned to the incubator. Neither growth in this medium nor the synchrony technique used significantly alters the kinetics of S-phase (Meyn et al., 1973). Cell samples were taken at various times and lysed prior to CsCl gradient analysis. The percentage of DNA replicated was determined by measuring the proportion of ^{14}C-radioactivity banding in the hybrid density region of preparative CsCl gradients. These gradients were prepared by mixing 4.0 ml of CsCl solution (63.8% wt/wt) and 0.7 ml of cell lysate. The gradients were centrifuged for 45 h at 33,000 rpm in a SW 50.1 rotor at 21°C. The gradients were fractionated and the ^{14}C-radioactivity in each fraction was determined by liquid scintillation counting. Generally, more than 90% of the counts put on the gradient were recovered in the fractions. In the subsequent figures, each data point will represent (except where noted) an individual determination. Experience has shown, however, that such determinations of the percent DNA replicated are usually reproducible within plus or minus 3%.

In the experiments designed to test for induction of a second round of DNA synthesis within the first S-phase after UV-irradiation, the cultures were pulse labeled with [^{3}H]TdR (5 μCi/ml, 6 Ci/mM, 0.1 μg/ml FUdR, and 1 μg/ml TdR) during the first 30 min of S-phase. The kinetics of replication of both the ^{14}C- and ^{3}H-labeled DNA were determined with BUdR as previously described.

RESULTS

The kinetics of semiconservative DNA replication in UV-irradiated CHO cells synchronized at the beginning of S-phase are shown in Fig. 1. After a small initial lag, the rate of DNA replication in the unirradiated cells is essentially uniform throughout

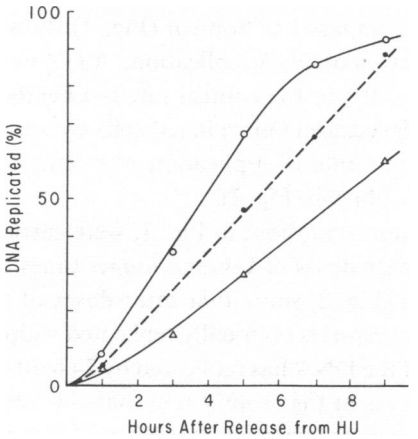

FIGURE 1 Effects of UV on DNA replication in synchronized CHO cells. [^{14}C]TdR-labeled cells were synchronized at the beginning of S-phase with 2mM hydroxyurea (HU) and irradiated. Immediately after irradiation the cells were incubated in medium containing 0.1 μg/ml FUdR and 50 μg/ml BUdR for various periods of time prior to CsCl gradient analysis. ○, unirradiated control; ●, cells which received 5 J/m^2; △, cells which received 10 J/m^2. Each data point represents an individual determination.

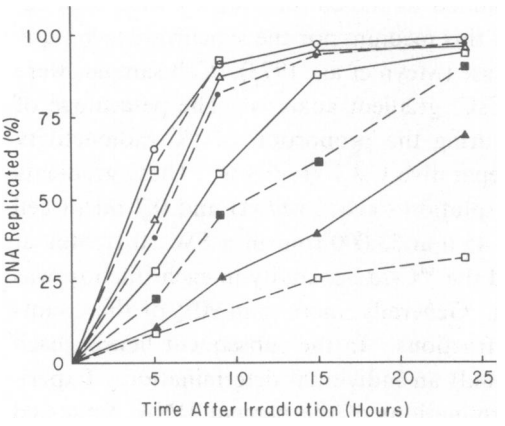

FIGURE 2

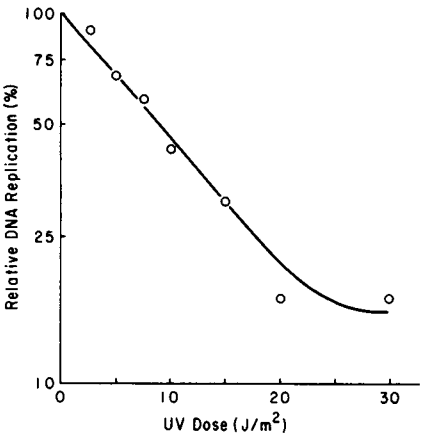

FIGURE 3

FIGURE 2 Effects of various doses of UV on DNA replication in synchronized CHO cells. Synchrony, irradiation, and incubation conditions were the same as in the legend to Fig. 1. ○——○, unirradiated control; □---□, 2.5 J/m^2; △——△, 5 J/m^2; ●——●, 7.5 J/m^2; □——□, 10 J/m^2; □- - -□, 15 J/m^2; ▲——▲, 20 J/m^2; □- - - -□, 30 J/m^2. Each data point, except for the points at 5 h, represents an individual determination. The points at 5 h are the average of two determinations.

FIGURE 3 The relative rate of DNA replication as a function of UV dose. The amounts of DNA replication at 5 h from Fig. 1 have been normalized to the unirradiated control and replotted in this figure. Each data point is the average of two determinations.

S-phase (7 h in these cells). The rate decreases after 6 h and achieves 95% total DNA replicated at 9 h. The UV-irradiated cultures show a dose-dependent reduction in the rate of DNA replication compared to control (Fig. 1); however, the irradiation did not induce a delay in initiation of DNA replication. A UV dose of 5 J/m^2 reduced the rate of DNA replication to 70% of the control rate but nearly the same extent of total DNA replication was achieved as in unirradiated cells by 9 h after irradiation (Fig. 1). After a dose of 10 J/m^2, the rate of replication was reduced such that only 60% of the DNA had replicated within 9 h (Fig. 1).

Further experiments, similar to those in Fig. 1, were carried out in order to extend the analysis to include higher doses of UV and longer times after irradiation. The results of these experiments (Fig. 2) show that after doses of up to 10 J/m^2 all of the DNA (within experimental error) is eventually replicated within 24 h. Even after doses as high as 15 J/m^2 90% of the DNA has replicated by 24 h after irradiation.

Examination of the curves in Fig. 1 indicated that the amount of DNA replicated by 5 h after irradiation was approximately proportional to the rate of DNA replication after irradiation. Therefore, the amounts of DNA replicated by 5 h after irradiation from Fig. 2 were normalized to the control and replotted as a survival curve of the rate of DNA replication as a function of the dose of UV (Fig. 3). This curve is essentially exponential up to a total dose of 20 J/m^2 with a slope (D_o) of 12.5 J/m^2. The tail on the curve at doses higher than 20 J/m^2 would indicate that some small fraction

(less than 20%) of DNA replication is more resistant to inhibition by UV. The biphasic nature of such curves has been observed by others (Cleaver, 1965; Cleaver, 1970; Rauth et al., 1974), but has not been satisfactorily explained. Since the cells were irradiated at a specific point in S-phase in the experiment of Fig. 3, it would seem that the biphasic response is not due to variations in the UV-sensitivity of cells in various parts of S.

Previous reports have indicated that UV-irradiation induces an aberrant type of DNA replication in bacterial cells characterized by a randomization of replication sequences (Hewitt and Billen, 1965; Hewitt et al., 1967). CHO cells were tested for a specific type of aberrant synthesis namely, induction of a second round of DNA replication within the same S-phase. [^{14}C]TdR labeled cells were first synchronized at the beginning of S-phase as in the previous experiments. In this case, however, the cells were then pulse labeled with [^{3}H]TdR for the first 30 min of S-phase. This pulse labeling period was followed by a chase with unlabeled TdR (10 μg/ml) for an additional 30 min in order to prevent mixing of [^{3}H]TdR and BUdR within the same segments of DNA. After the chase period, the cells were irradiated and the kinetics of replication of both ^{14}C- and ^{3}H-labeled DNA were determined with BUdR as before. The radioactivity profiles of two of the CsCl gradients from this experiment are shown in Fig. 4. These gradients show the relative replication of both the ^{14}C-labeled parental

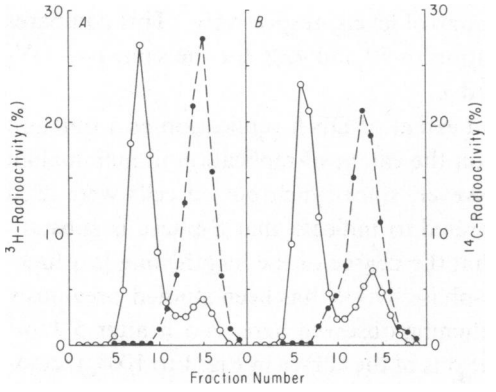

FIGURE 4

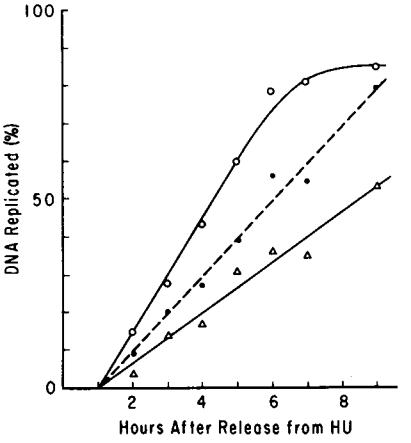

FIGURE 5

FIGURE 4 CsCl density gradient profiles. [^{14}C]TdR-labeled cells were synchronized at the beginning of S-phase with HU and then pulse labeled for 30 min with [^{3}H]TdR. This was followed by a 30 min chase period with unlabeled TdR and then irradiation. Immediately after irradiation the cells were incubated in medium containing FUdR and BUdR for 8 h prior to CsCl gradient analysis. (A) Unirradiated control. (B) Cells which received 5 J/m^2. ○, ^{14}C-counts; ●, ^{3}H-counts.

FIGURE 5 Effects of UV on DNA replication in synchronized CHO cells irradiated 1 h into S-phase. Synchrony and irradiation conditions were the same as in the legend to Fig. 4. Immediately after irradiation the cells were incubated in medium containing FUdR and BUdR for various periods of time prior to CsCl gradient analysis. ○, unirradiated controls; ●, cells which received 5 J/m^2; △, cells which received 10 J/m^2. Each data point represents an individual determination.

and ^{3}H-pulse labeled DNA at 8 h after irradiation for both the unirradiated control and the population of cells which received 5 J/m^2. In both cases, the majority of the parental DNA (^{14}C-counts) has been replicated, but in neither case has any ^{3}H-labeled DNA been replicated. Qualitatively similar results (not shown) were found for cells irradiated with 10 J/m^2. Thus, UV-irradiation does not induce premature initiation of new rounds of DNA synthesis in CHO cells. The complete kinetics of the replication of the ^{14}C-labeled parental DNA are shown in Fig. 5. Comparison of these curves with those of Fig. 1 show that there is little difference in the effect of UV-irradiation on the rate of DNA replication when the cells are irradiated 1 h into S-phase as compared to irradiating at the G1-S boundary.

DISCUSSION

Two characteristics of this investigation must be taken into consideration when comparing the results with previous reports. First, the techniques used for measuring rates of DNA replication are quite different from those usually employed and actually yield different types of information. Second, in the experiments reported here, synchronized cells were irradiated at specific points in the cell cycle. The effects of UV have been studied in synchronized cells to a lesser extent than in asynchronous cells. In spite of these differences our results are both qualitatively and quantitatively similar to the findings of others. For example, Domon and Rauth (1968) have reported that 4 and 10 J/m^2 reduced [^{3}H]TdR incorporation, measured 2 h after irradiation in asynchronous mouse L cells, to 46 and 29% of control levels, respectively. This compares to the reduction in the rate of DNA replication to 70 and 43% for the same two UV-doses, respectively, as shown in Figs. 1, 2, and 3.

The data in Figs. 1 and 2 show the rate and extent of DNA replication as a function of time after irradiation. The effects of UV on the extent of replication in mammalian cells have not been studied previously. However, since synchronized cells were used in these experiments, the data can be interpreted to indicate that S-phase is substantially lengthened after UV-irradiation and that the degree of the lengthening is a function of the UV dose. The lengthening of S-phase by UV has been studied previously by a number of investigators, and the lengthening observed here, 2–3 h after 5 J/m^2 (estimated by extrapolation of the linear portions of the curves in Fig. 1 to 100%), compares well with the 1–2 h delay after a dose of 4 J/m^2 reported by Domon and Rauth (1968) for mouse L cells.

Fig. 1 shows that after a dose of 5 J/m^2, DNA replication is considerably slowed, but ultimately as much DNA is replicated as is in control cells. After relatively high doses of UV (15 J/m^2) nearly all of the DNA is eventually replicated within 24 h after irradiation (Fig. 2). Because of limits in the precision of these data we cannot rule out the possibility that some small portion of the genome (less than 5%) is never replicated even at the smaller doses. However, if one assumes that the average replicon size is about 30 μm (Gautschi et al., 1973) and that the fraction of [^{3}H]thymidine in dimers is 5×10^{-5} per J/m^2 (Meyn et al., 1974) one can calculate (Setlow et al., 1969) that a dose of 15 J/m^2 will produce on the average about 14 dimers per replicon. Therefore,

an important conclusion from these findings is that most, if not all, of the DNA can be replicated after UV-irradiation in spite of the presence of many pyrimidine dimers per replicon. We have previously shown that at least 65% of the dimers produced by 5 J/m^2 are still present 24 h after irradiation in these cells (Meyn et al., 1974). Thus, it is not clear what role (if any) dimers play in limiting either the size of the DNA synthesized after UV-irradiation or its rate of synthesis (Hewitt and Meyn, 1975). It is clear, however, that dimers cannot be absolute blocks to DNA replication. In order to account for the nearly total genome replication reported here, a mechanism must exist that allows genome replication between dimers even after UV doses that produce many dimers per replicon. Two such mechanisms have been proposed. In the first, mammalian cells are assumed to bypass UV-induced photoproducts during DNA replication by a process similar to that proposed for bacterial cells by Rupp and Howard-Flanders (1968). In mammalian cells, however, the gaps left in the newly synthesized DNA are apparently filled by *de novo* synthesis rather than by sister-strand exchanges (Lehmann, 1972). A second mechanism suggested by Painter (1974) assumes that dimers or other photoproducts may act as blocks to DNA synthesis for relatively long periods of time. These blocks are later bypassed allowing adjacent incompletely replicated replicons to be completed and the newly synthesized strands can then be joined to form fully completed DNA molecules. The observation of nearly complete genome replication after doses of UV which produce many dimers per replicon (Fig. 2) supports either of these proposed mechanisms and is a necessary consequence of either model which has not been previously demonstrated.

In previous experiments designed to determine the time course of bypass of photoproducts during DNA synthesis, we observed that UV-irradiated cells began to synthesize DNA of the same size as unirradiated cells within 6 h after irradiation (Meyn and Humphrey, 1971). Our interpretation of those results was that an additional repair process restored the template DNA during the first 6 h after irradiation such that normal molecular weight DNA could by synthesized. Similar observations have been made in normal human cells and in cells from patients having the disease Xeroderma pigmentosum (XP) by Buhl et al., (1973). Lehmann and Kirk-Bell (1972) reported this phenomenon in mouse L5178Y cells and suggested two alternative interpretations. First, that at later times after irradiation the bypass enzymes are more efficient which results in a rapid filling of gaps; or second, that aberrant DNA synthesis, such as premature initiation of new round of synthesis within the same S-phase, may be induced by UV. The first of these two possibilities is in accordance with the findings of Chiu and Rauth (1972) in UV-irradiated mouse L cells. They could not detect gaps after low doses and the gaps that were detected after higher doses were at a lower frequency than expected. However, evidence provided by Buhl et al. (1973) seems to rule out the possibility of rapid gap-filling. The second of these possibilities, UV-induced alteration of DNA replication sequence, was tested in the experiment presented in Fig. 4. The results show that new rounds of DNA replication within the same S-phase are not induced by UV. Such aberrant synthesis had been reported for UV-irradiated bacterial cells (Hewitt and Billen, 1965, and Hewitt et al., 1967), but a recent reexamina-

tion of this question yielded an alternative interpretation of the original data (Hewitt and Gaskins, 1971). Buhl et al. (1973) have also examined this question in mammalian cells and have shown that the DNA synthesized at long times after irradiation is made from the DNA template that had been irradiated.

The survival curve of DNA replication versus UV-dose (Fig. 3) displayed a D_o (the dose to reduce survival to 37% along the exponential portion of the survival curve) of 12.5 J/m^2. This is in contrast to the D_o characteristic of CHO cell survival which is about 2 J/m^2 (Humphrey et al., 1970). This apparent lack of correlation between the effects of UV on DNA replication and on cell survival confirms a similar observation by Rauth et al. (1974). They found that the ability of UV-irradiated HeLa, L, or CHO cells to synthesize DNA as measured by [^{3}H]thymidine uptake was similar, as was the production of thymine dimers. The colony forming abilities of the three cells lines differed, however, and a correlation was found between the frequency of small segments of newly synthesized DNA and cell killing.

An additional observation that can be made from the data presented in Figs. 1 and 5 is that the reduction in the rate of DNA replication produced by UV-irradiation either at the G1-S boundary (Fig. 1) or 1 h into S-phase (Fig. 5) is the same. Cell sensitivity, on the other hand, as measured by colony-forming ability is greatly reduced in early S-phase (Humphrey et al., 1970). Provided that the damage to DNA at these times is similar, these findings together with those of Rauth et al. (1974) suggest that the rate of DNA replication after UV reflects the degree of damage to DNA, whereas the cell survival after UV reflects the degree of repair of that damage. This idea is further substantiated by the fact that the UV-induced depression in the rate of DNA synthesis in normal human fibroblasts and in cells derived from XP patients is identical (Cleaver, 1970).

In conclusion, the data presented here lend further support to the idea that mammalian cells can bypass UV-induced photoproducts in their DNA during DNA replication. However, it is clear that before the details of such a process can be completely understood, the role that pyrimidine dimers and/or other photoproducts play in reducing the size of DNA synthesized or the rate of DNA replication must be fully assessed.

The authors wish to acknowledge the technical assistance of Mrs. B. A. Sedita and Miss J. M. Winston.

This work was supported by U.S. Public Health Service research grant CA 04484 from the National Cancer Institute. Laurence F. Thomson is a predoctoral trainee supported by U.S. Public Health Service grant CA 05099.

Received for publication 7 July 1975 and in revised form 25 September 1975.

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