RATE OF DNA CHAIN ELONGATION IN ULTRAVIOLET LIGHT-IRRADIATED MAMMALIAN CELLS AS ESTIMATED BY A BROMODEOXYURIDINE PHOTOLYSIS METHOD

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ABSTRACT Using a new method, we show that, when the DNA chain elongation rate is measured for short time periods, ultraviolet light does not decrease this rate. The method is based on the photolysis of bromouracil-containing DNA by 313 nm light and alkaline sucrose gradients.

INTRODUCTION

Exposure of cultured mammalian cells to large fluences of ultraviolet (UV) light (10 J/m^2) inhibits DNA synthesis. In some models of post-UV replication, it has been assumed that this inhibition is due to a decreased rate of chain elongation (Lehmann, 1972). It is difficult to determine the rate of chain elongation after UV irradiation by autoradiography or density gradient techniques because the lengths of DNA formed during the UV-induced inhibition of DNA synthesis are too short to be measured accurately by these methods. With photolysis of DNA containing bromodeoxyuridine (BrdUrd) and equations derived here, however, chain elongation rates during a short (3–4 min) radioactive BrdUrd pulse can be determined. In such experiments no decrease in the rate of chain elongation could be detected after UV-irradiation. This result is consistent with a hypothesis based on a temporary decrease in the number of growing points as elongating chains encounter lesions in the template strand (Klímek and Vanícek, 1970; Edenberg, 1973).

MATERIALS AND METHODS

HeLa S3 or CHO cells, maintained in this laboratory for several years, were grown on Falcon plastic petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in Eagle's minimum essential medium (MEM) containing 15% fetal calf serum (Gibco Diagnostics, Chagrin Falls, Ohio).

Exponentially growing cells were incubated overnight in 0.001 μCi/ml of [^{14}C]thymidine (57 Ci/mol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.). They were then washed twice with Puck's phosphate-buffered saline G, exposed to various fluences of UV light (0, 6.5, or 11 J/m^2) from a mercury germicidal lamp with an incident intensity of 1.3

$J/m^2 \cdot s$, and incubated in MEM for 30 min to allow expression of UV-induced inhibition of DNA synthesis. Cells were then pulse-labeled in warmed 5% CO_2-equilibrated medium containing 50 μCi/ml of [^{3}H]thymidine (3×10^{-6} M), BrdUrd (10^{-5} M), and fluorodeoxyuridine (10^{-6} M) (to inhibit *de novo* thymidine synthesis). The reaction was stopped by rinsing the cells with ice-cold SSC (0.15 M sodium chloride, 0.015 M sodium citrate) containing 30 mM KCN and 1 mM thymidine, and the cells were scraped into ice-cold SSC.

To determine [^{3}H]thymidine incorporation, an aliquot of these cells was sonicated and its DNA was precipitated onto Whatman GF/C glass fiber filters by adding an equal volume of 1.5 N HCl containing 6% $Na_4P_2O_7 \cdot 10\ H_2O$. The filters were washed with ice-cold 4% perchloric acid and 70 and 100% ethanol and dried. Radioactivity in the filters was counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.), using toluene containing Omnifluor (New England Nuclear, Boston, Mass.). Overall DNA synthesis rates were determined from the ratios of ^{3}H to ^{14}C counts.

Other aliquots of the cells, to be used for determination of chain elongation rates, were exposed to various fluences of 313 nm light. The procedure for irradiation at 313 nm was exactly as described previously (Povirk and Painter, 1976). The output of a Phillips SP-500 high pressure mercury-arc lamp was filtered through 2.3 cm thicknesses of each of the following solutions: (*a*) distilled water; (*b*) 3.5×10^{-4} M BrdUrd in 0.1 N HCl; (*c*) 4.4×10^{-4} M $K_2Cr_2O_7$ in 0.1 N NaOH; and (*d*) 87 g/liter of $CoCl_2 \cdot 6\ H_2O$, 44 g/liter of $NiCl_2 \cdot 6\ H_2O$, and 1 M HCl dissolved in 55% dimethylformamide in water. The resulting intensity was between 20 and 30 $J/m^2 \cdot s$. The DNA from these cells was analyzed on alkaline surcose gradients as described before (Povirk and Painter, 1976). The frequency of strand breaks in labeled segments of the DNA was determined by the changes in sedimentation patterns caused by 313 nm light (see Appendix). Calibration of 313 nm-induced strand breaks was similar to that described by Lehmann and Ormerod (1970) and Lehmann (1972), except that all *S* values were corrected by a factor of 1/1.09. This was done because experiments with mechanically sheared DNA showed that BrdUrd-substituted strands, due to their higher density, sediment 9% faster than unsubstituted strands of the same length (data not shown). The calibration indicated a single-strand break frequency of 7.1×10^{-11} breaks/(dalton $\cdot$ J/m^2) at fluences of 0–2,000 J/m^2.

RESULTS

When cells were pulse-labeled with [^{3}H]thymidine and BrdUrd, the radioactive label was distributed over a wide range of molecular weights, indicating that short, radioactive BrdUrd-containing segments had been added to the ends of longer growing DNA chains (see Fig. 1). When cells were exposed to 313 nm light after termination of the pulse, there was a loss of radioactivity from molecules with molecular weights greater than 2×10^7 daltons and a corresponding increase in radioactivity in molecules of less than 10^7 daltons (Fig. 2 *a*). This change was presumably the result of strand breaks in the BrdUrd-containing portions of the DNA (Hutchinson, 1973), because decreases in the overall length of the growing DNA chains were not sufficient to account for it. Assuming a normal chain elongation rate (0.8×10^6 daltons/min), loss of BrdUrd-containing segments from the ends of growing molecules would change their molecular weight by, at most, 7×10^6 daltons, a change of less than one gradient fraction even for the smallest molecules being considered. Control experiments, using a pulse of [^{3}H]thymidine only, indicated that 313 nm-induced breaks in unsubstituted portions of the chains had no measurable effect on the shape of the sedimentation profiles (Fig.

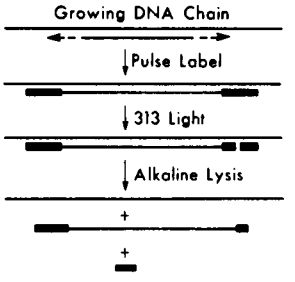

FIGURE 1

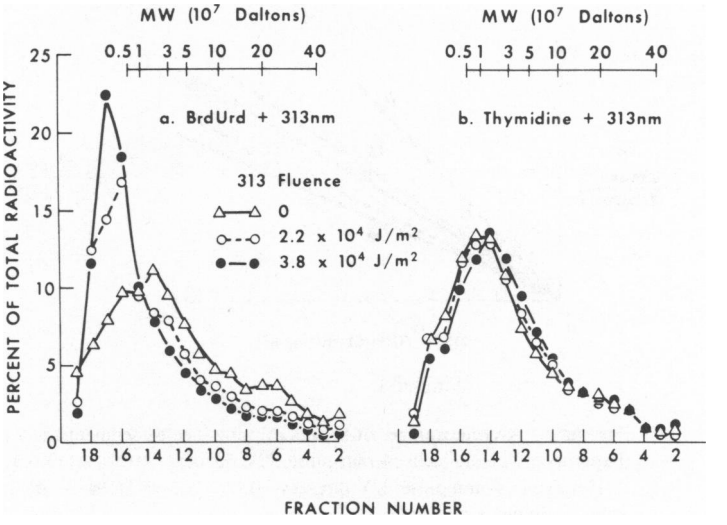

FIGURE 2

FIGURE 1 Experimental rationale. The ends of growing DNA chains are labeled with [^{3}H]thymidine and BrdUrd. Subsequent exposure to 313 nm light breaks off portions of these labeled segments. The fraction remaining attached to longer DNA strands after alkaline lysis is a function of the segment length and can be used to determine the elongation rate. (——) normal DNA; (▬) radioactive BrdUrd-containing DNA.

FIGURE 2 Alkaline sucrose gradients of DNA from unirradiated HeLa cells pulse-labeled with (*a*) [^{3}H]thymidine and BrdUrd or (*b*) [^{3}H]thymidine only for 4 min and subsequently exposed to 0, 22,000, or 38,000 J/m^2 of 313 nm light. Similar patterns were observed in profiles from irradiated cells, although a smaller portion of the radioactivity was found in the high molecular weight region of the gradients.

2*b*). Thus, the loss of small labeled segments from the ends of the larger growing chains must account for the majority of this shift in radioactivity.

In order to estimate the rate of DNA chain elongation, it was necessary to calculate the fraction (f) of radioactivity remaining attached to the large molecules after exposure to 313 nm light. This is the portion of radioactivity found in fractions 2–12 (molecular weight > 3×10^7 daltons) of gradients from BrdUrd-containing cells exposed to 313 nm light divided by the portion of radioactivity (15–40%) found in the same fractions of gradients from unexposed cells. The cutoff point of 3×10^7 daltons was chosen to insure adequate separation from the peak of small fragments.

The average number of strand breaks per pulse-labeled segment (Q) was calculated from f with the following equation (see Appendix): $f = [1 - \exp(-Q)]/Q$. As expected, there was an approximately linear relationship between 313 nm exposure and number of breaks per segment (Fig. 3). The number of breaks per segment was about the same in UV-irradiated and in control cells, indicating that, if the degree of BrdUrd substitution was the same, the segment lengths, and thus the rates of elongation, were the same in both cases. The results of incorporation experiments (Fig. 4) show that the degree of BrdUrd substitution was indeed the same: in a 4 min pulse, the degree of sub-

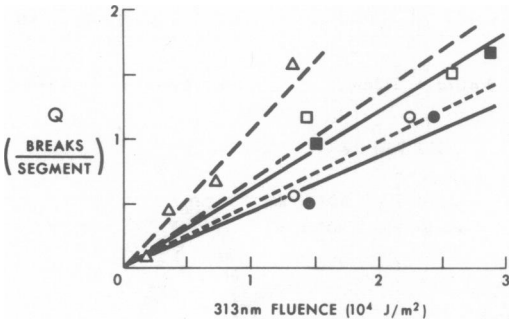

FIGURE 3

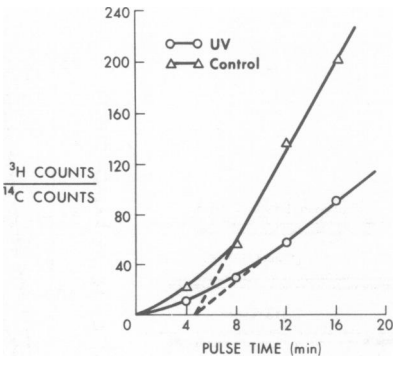

FIGURE 4

FIGURE 3 Average number of breaks in pulse-labeled segments as a function of 313 nm fluence. Experiment 1: HeLa cells, 4 min pulse, UV fluences—0 (○- - -○) or 6.5 (●——●) J/m^2. Experiment 2: HeLa cells, 4-min pulse, UV fluences—0 (□- - -□) or 11 (■——■) J/m^2. Experiment 3: CHO cells, 3 min pulse, no UV (△- - -△).

FIGURE 4 Incorporation of [^{3}H]thymidine and BrdUrd into acid-precipitable material. Cells were incubated overnight in 0.001 μCi/ml of [^{14}C]thymidine, exposed to 0 or 6.5 J/m^2 of UV light, incubated for 30 min, and pulsed with 50 μCi/ml of [^{3}H] thymidine (3×10^{-6} M), 10^{-5} M BrdUrd, and 10^{-6} M fluorodeoxyuridine. If the rate of chain elongation does not vary during the pulse, the rate of increase of the ^{3}H to ^{14}C ratio is a measure of the fraction of thymines in newly made DNA derived from exogenous precursors. Thus, assuming this fraction is 100% during the time corresponding to the linear portion of the curve, the average degree of exogenous substitutions in the first 4 min was: [incorporation in the first 4 min/(linear rate × 4 min)] = 10.1/(8.4 × 4) = 0.301 for UV-irradiated cells and 21.2/(17.5 × 4) = 0.303 for controls.

stitution by exogenous precursors was about 0.30 for both irradiated and control cells. Because the exogenous pool included 1.0×10^{-5} M BrdUrd and 0.3×10^{-5} M thymidine, the degree of BrdUrd substitution was $b/b_0 = 0.30[1.0/(1.0 + 0.3)] = 0.23$. The length of DNA synthesized during the pulse (see Appendix) was $L = QM_n b_0/b$, where Q and M_n (the number-average molecular weight of the segments produced from fully BrdUrd-substituted DNA) are determined for each fluence, as described in the Materials and Methods. The results of this calculation (Table I) show that the DNA chain elongation rate in HeLa cells decreased only slightly, if at all, after UV irradiation, and not nearly enough to account for the inhibition of total DNA synthesis.

To validate these measurements, we used our method to determine the rate of chain elongation in CHO cells (Table I), and we obtained results similar to those obtained by other means (Painter and Schaefer, 1969; Gautschi and Kern, 1973; Edenberg and Huberman, 1975). We also determined the elongation rate in HeLa cells in the presence of cycloheximide (10 μg/ml) and found a depression of thymidine incorporation to 18% of controls accompanied by a decrease in chain elongation rate to 26% of controls. Using density gradient methods and CHO cells under similar conditions, Gautschi and Kern (1973) found a depression of incorporation to 19% accompanied by a decrease in chain elongation rate to 20%. These data also indicate that the BrdUrd photolysis method used here provides a valid measure of the rate of chain elongation.

TABLE I

ESTIMATION OF TOTAL DNA SYNTHESIS ([^{3}H]THYMIDINE UPTAKE) AND DNA CHAIN ELONGATION RATES AFTER UV IRRADIATION

Cell line	Pulse time	UV fluences	Inhibition of [^{3}H]thymidine uptake	Average QM_n^* ($\times 10^5$)	b/b_0	Length of DNA synthesized during the pulse	Rate of chain elongation
	min	J/m^2	%			*μm*	*μm/min*
HeLa	4	0	—	6.90	0.23	3.0	0.8
		6.5	52	6.07	0.23	2.6	0.7
HeLa	4	0	—	9.44	0.23	4.1	1.0
		11	76	8.55	0.23	3.7	0.9
CHO	3	0	—	15.7	0.41	3.8	1.3

*Determined from the slopes in Fig. 2 with the relation $1/M_n$ = (fluence) · [7.1 × 10^{-11} breaks/(dalton · J/m^2)].

DISCUSSION

The molecular basis for inhibition of DNA synthesis after UV irradiation is uncertain. There is much indirect evidence supporting the proposal that pyrimidine dimers interrupt DNA chain growth. In several cell lines (Cleaver and Thomas, 1969; Buhl et al., 1972*a*; Fujiwara and Kondo, 1972; Lehmann, 1972), DNA that is made 30 min after exposure to UV light has approximately the same mean length as the distance between dimers in the parental strands. In addition, the rate of [^{3}H]thymidine incorporation during the first few hours after irradiation is inversely proportional to UV fluence and thus is directly proportional to the distance between dimers (Edenberg, 1973). Furthermore, we found that the time needed for the rate of uptake to decrease to a stable level after exposure to 10–20 J/m^2 of UV light was of the same order as the average time that growing chains would be expected to continue before encountering a dimer (data not shown). Although our data show no decrease in the chain elongation rate between dimers, they do not rule out the possibility that blocks occur at average distances greater than those traveled by the growing points during the pulse times used in our method. Thus, our data do not refute the possibility that dimers act as temporary blocks to DNA replication. The work of Lehmann (1972) and of Buhl et al. (1972*b*), however, indicated that low molecular weight chains made after UV irradiation are separated from longer growing strands only by short gaps (about 1,000 nucleotides); this result has led to the suggestion that the replication machinery simply skips over the dimer and continues replicating beyond it. If this is the case, some other explanation must be invoked to account for inhibition of DNA synthesis, and our data indicate that it is not a change in the elongation rate of those DNA chains which are actually growing at any given time. One possibility is that the elongation machinery does in fact skip over dimers but suffers delays of at least several minutes, either at the dimers or at other UV-induced lesions having comparable rates of formation.

APPENDIX

Equations to Determine the Rate of DNA Chain Elongation

Consider a long DNA molecule substituted with BrdUrd near the ends only (Fig. 1). Let p be the probability that a single BrdUrd residue will produce a strand break at a given fluence of 313 nm light. Number the BrdUrd residues from 1 to N, starting at the point where the pulse-labeled segment was attached to the longer growing DNA chain (see Fig. 1). The probability that the first break occurs at the nth residue is $P_n = p(1 - p)^{n-1}$, and the amount of radioactivity remaining attached is $R_n = nr$, where r is the amount of radioactivity associated with each BrdUrd residue. (r is a constant because [^{3}H]thymidine and BrdUrd follow the same metabolic pathways in the cell.) The probability that no breaks occur is $P_0 = (1 - p)^N$, in which case the total radioactivity, Nr, remains attached. Thus, in a large population, the average fraction of the radioactivity remained attached is

$$f = \left[\sum_{n=1}^{N} P_n R_n + rNP_0\right] \Big/ Nr = \left[\sum_{n=1}^{N} nrp(1 - p)^{n-1} + Nr(1 - p)^N\right] \Big/ Nr.$$

Since p is small, $(1 - p)^n \simeq \exp(-pn)$ and

$$f \simeq \left[pr \int_0^N n\exp(-pn)\,dn + rN\exp(-pN)\right] \Big/ Nr$$

$$= [1 - \exp(-pN)]/pN = [1 - \exp(-Q)]/Q,$$

where $Q = pN$ is the average number of breaks per labeled segment. f is determined experimentally, and a value of Q is interpolated from a table of f vs. Q.

To determine p, a calibration of strand breaks was done with very long, uniformly labeled, fully substituted DNA molecules. If these have b_0 BrdUrd residues per unit length, then the number-average molecular weight (in daltons) of pieces formed by a given fluence of 313 nm light is $M_n = 1/b_0p$, or $p = 1/b_0M_n$. M_n which can be determined from sucrose gradients, was inversely proportional to fluence.

Because it takes a finite time for BrdUrd to saturate precursor pools, the pulse-labeled DNA will not be fully substituted. It will have an average of $b = N/L$ residues per unit length, where L is the length of the pulse-labeled segment. Thus, at a given fluence of 313 nm light, $L = N/b = pN/pb = QM_nb_0/b$; b/b_0 is the fraction of thymidine residues in the pulse-labeled segment that have been substituted with BrdUrd, a quantity which can be determined from incorporation experiments.

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