REPLICON SIZES IN MAMMALIAN CELLS AS ESTIMATED BY AN X-RAY PLUS BROMODEOXYURIDINE PHOTOLYSIS METHOD

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ABSTRACT A new method is described for estimating replicon sizes in mammalian cells. Cultures were pulse labeled with [^{3}H]thymidine ([^{3}H]TdR) and bromodeoxyuridine (BrdUrd) for up to 1 h. The lengths of the resulting labeled regions of DNA, L_{obs}, were estimated by a technique wherein the change in molecular weight of nascent DNA strands, induced by 313 nm light, is measured by velocity sedimentation in alkaline sucrose gradients. If cells are exposed to 1,000 rads of X-rays immediately before pulse labeling, initiation of replicon operation is blocked, although chain elongation proceeds almost normally. Under these conditions L_{obs} continues to increase only until operating replicons have completed their replication. This value for L_{obs} then remains constant as long as the block to initiation remains and represents an estimate for the average size of replicons operating in the cells before X-irradiation. For human diploid fibroblasts and human HeLa cells this estimated average size is approximately 17 μM, whereas for Chinese hamster ovary cells, the average replicon size is about 42 μM.

INTRODUCTION

Replicons in cultured eukaryotic cells have been measured by a number of workers, and estimates for their sizes range from 2.54 to 100 μM (Edenberg and Huberman, 1975). The estimates for small replicons in *Drosophila* embryo DNA were made by electron microscopy (Blumenthal et al., 1973); most estimates, however, were arrived at by autoradiography (Edenberg and Huberman, 1975). Although autoradiography may be the most accessible method, it very possibly overestimates replicon sizes because the resolution of the techniques makes it very difficult to detect small replicons (Huberman and Riggs, 1968).

Replicon sizes have also been estimated by using alkaline sucrose gradients and cesium chloride gradients (for review, see Edenberg and Huberman, 1975). Roti Roti and Painter (1977) recently have generated equations that make it possible to calculate average replicon sizes from CsCl density gradient procedures. They calculated a value of 13 μM for WI-38 cells and 6.5 μM for HeLa cells.

Initiation of mammalian replicons is inhibited by relatively low doses of X-rays (Makino and Okada, 1975; Walters and Hildebrand, 1975; Painter and Young, 1975). Using this fact plus a recently developed method for measuring the rate of DNA chain elongation (Povirk and Painter, 1976*a*), we obtained a value for replicon sizes in the

following way. Cells are exposed to 1,000 rads of X-rays and then immediately incubated for various lengths of time with [^{3}H]thymidine ([^{3}H]TdR) and bromodeoxyuridine (BrdUrd). The length of the average segment of DNA labeled with tritium, L_{obs}, can be estimated from the changes induced in the profiles of alkaline sucrose gradients by 313 nm light, as described by Povirk and Painter (1976*a*). In normally growing cells, L_{obs} increases continuously with the length of the pulse label. However, in X-irradiated cells in which initiation events are blocked, only replicons that had initiated before irradiation continue to elongate; L_{obs} grows only until these replicons have ceased to operate. Inasmuch as almost no new initiations take place, L_{obs} is constant until recovery of initiation occurs. Thus, this value of L_{obs} should represent an average value for the size of replicons in operation during the time of exposure to X-rays. Using this method, we estimated the average size of HeLa replicons and of human diploid fibroblast replicons to be about 17 μM, and the average size of Chinese hamster ovary (CHO) cell replicons to be about 42 μM.

METHODS

Three cell lines were used: CHO cells maintained in McCoy's 5A medium, HeLa cells maintained in Eagle's minimum essential medium, and diploid fibroblast E-11 cells from a human foreskin cell line established by A. Millis (State University of New York, Albany) and maintained in medium 199 (Microbiological Associates Inc., Bethesda, Md.). All media contained 15% fetal calf serum, penicillin (50 U/ml), streptomycin (50 U/ml), chlortetracycline (50 μg/ml), and 10 mM Hepes buffer. The generation times were approximately 14 h for CHO cells and 20 h for HeLa and E-11 cells.

Alkaline sucrose gradient centrifugation was carried out as described by Povirk and Painter (1976*a*). After being labeled, cells were washed three times in cold SSC (0.15 M sodium chloride, 0.015 M sodium citrate), scraped off Petri dishes, and exposed to 1,200 rads of X-rays to enhance lysis (Wheeler et al., 1974). 0.5 ml of cells was then placed onto 0.5 ml of lysis solution (0.2 M NaOH, 0.02 M EDTA, 0.1% nonidet NP·40 [Shell Oil]) on top of a 36-ml 5–20% alkaline sucrose gradient (0.1 M NaOH, 0.9 M NaCl, and 0.02 M EDTA). Gradients were centrifuged at 27,000 rpm for 2.5 h at 20°C in a Beckman SW27 rotor (Beckman Instruments, Inc., Fullerton, Calif.), and collected in 10 or 20 fractions from the bottom of the tubes. Carrier DNA was added to each fraction and DNA precipitated with 6% pyrophosphate in 1.5 N HCl. The precipitates were collected on Whatman GF/C glass fiber filters (Whatman Inc., Clifton, N.J.) and washed with cold 4% $HClO_4$ and 70, 95, and 100% ethanol. Filters were dried and counted in toluene containing Omnifluor (New England Nuclear, Boston, Mass.) in a liquid scintillation counter.

For X-irradiation a G.E. Maxitron 300 (General Electric Co., Milwaukee, Wis.) was used at 300 rads/min. Light at 313 nm was produced by a Philips SP500 high pressure mercury arc lamp (Philips Electronic Instruments, Inc., Mahwah, N.J.) filtered through 2.3 cm of each of the following four solutions (Povirk and Painter, 1976*b*): (*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 M NaOH; (*d*) 87 g/liter $CsCl_2 \cdot 6H_2O$, 44 g/liter $NiCl_2 \cdot 6H_2O$, 1 N HCl dissolved in 55% dimethyl formamide, 45% water (Wladmiroff, 1966). The solutions were in Vycor flasks (Corning Glass Works, Corning, N.Y.), which permit transmission of light with wavelengths of 260 nm and longer. The intensity was monitored with a Kettering model 65 radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio).

The method used to estimate the size of the labeled piece of DNA during a pulse label is that described by Povirk and Painter (1976*a*). Cells were pulse labeled with [^{3}H]TdR

(50 μCi/ml, 57 Ci/mM), BrdUrd (10^{-5} M), and fluorodeoxyuridine (FUrd) (10^{-6} M). The pulse label was stopped by washing the cells in ice-cold SSC. The cells were then scraped off the Petri dishes, and each sample split into three fractions, which were then exposed for 0, 1, or 3 min, respectively, to 313 nm light. The cells were analyzed by alkaline sucrose gradient centrifugation as described above. The tritium distributions in the sucrose gradient of the unexposed samples served as a control; the tritium distributions in the exposed samples shifted progressively toward the top of the gradients with increasing 313-nm fluence. The length of the labeled piece of DNA is $L = QM_n b_0/b$ (Povirk and Painter, 1976*a*), where Q is the average number of breaks per labeled segment; M_n can be determined from a calibration curve and is the number average molecular weight (in daltons) of pieces formed by a given fluence of 313 nm light; b/b_0 is the fraction of thymine residues in the pulse-labeled segment that have been substituted with BrdUrd. Each of these quantities can be determined experimentally and L then calculated as described in detail by Povirk and Painter (1976*a*).

As an example, using sucrose gradients such as those shown in Fig. 2,

$$r = [\text{cpm from fractions 1-12}]/[\text{total cpm in gradient}],$$

can be calculated for each gradient. For each gradient that was exposed to a 313-nm fluence, $f = r$ for that gradient/r for control gradient (no 313-nm exposure). It can be shown that $f = (1/Q)[1 - e^{-Q}]$ (Povirk and Painter, 1976*a*). With this relationship, each value of Q generates a value of f. Thus, any experimental value of f corresponds to a value of Q which can be read from a table. Next, it is necessary to obtain a value for $M_n \cdot M_n$ which is inversely proportional to the 313-nm fluence. A graph of $1/M_n$ vs. fluence is a straight line that holds for the range of fluences used in the experimental conditions described here (Lehmann, 1972). Since $1/M_n$ is proportional to fluence, [slope] · [fluence] = $1/M_n$ and $M_n = 1/$[slope] · [fluence]. The slope of this graph is constant. The fluence for each 313-nm exposure was measured with a Kettering radiometer (Yellow Springs Instruments) during each experiment. The final form of the equation used to calculate L_{obs} is

$$L_{obs} = \frac{Q}{[\text{slope}]\cdot[\text{fluence}]} \cdot \frac{1}{b/b_0}$$

The method used to calculate b/b_0 is described in the legend to Fig. 1. Thus each parameter was found experimentally, making it possible to calculate L_{obs}. The derivation of the equation for L_{obs} is shown in detail in Povirk and Painter (1976*a*).

RESULTS

The basic idea of these experiments is to measure the length of a pulse-labeled segment of DNA, which is at the end of the daughter strand molecule in a growing replicon. This can be accomplished in the following way. The [^{3}H]TdR is mixed with a 10-fold excess of BrdUrd, and both are incorporated into the DNA. The presence of BrdUrd residues sensitizes the pulse-labeled segment to photolysis (strand breakage) by 313 nm light. After a given fluence of 313 nm light, a given average amount of the pulse-labeled segment will remain attached to the unlabeled part of the daughter strand. At the fluences used, photolysis occurs in only a fraction of the BrdUrd residues. Therefore, the amount of radioactivity remaining attached to large unlabeled DNA segments after 313 nm irradiation allows the size of the pulse-labeled segment to be calculated. Thus, by determining the distribution of tritium on alkaline sucrose

gradients, one can determine the length of the labeled segment using the equations described by Povirk and Painter (1976*a*). To use these equations, several quantities must be measured as described in Methods. These are f, 313-nm fluence, and b/b_0.

Exogenously added [^{3}H]TdR and BrdUrd require time to enter the cellular pools and to be incorporated into the DNA. To estimate the extent of substitution of BrdUrd for TdR in the labeled DNA, E-11 cells were prelabeled overnight with [^{14}C]TdR (0.005 μCi/ml) and then exposed to [^{3}H]TdR and BrdUrd and FUrd for various times, fixed, and counted, and the ^{3}H/^{14}C ratios calculated as a function of labeling time (Fig. 1). The pool equilibration time was approximately 8 min. The degree of BrdUrd substitution into the labeled piece of DNA, b/b_0, was calculated from this graph as explained in the legend to Fig. 1 and in Povirk and Painter (1976*a*). Similar procedures were used to determine b/b_0 for HeLa and CHO.

From a set of gradients (Fig. 2) and the data from the labeling curves of Fig. 1, the length, L_{obs}, of the piece of DNA synthesized during the pulse label time can be calculated as described in Methods. In addition, the rate of chain elongation can be calculated as: (L_{obs}/duration of pulse label).

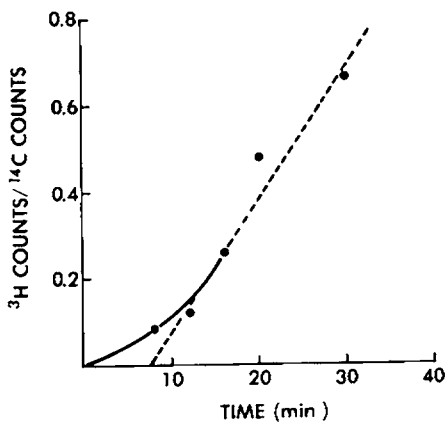

FIGURE 1

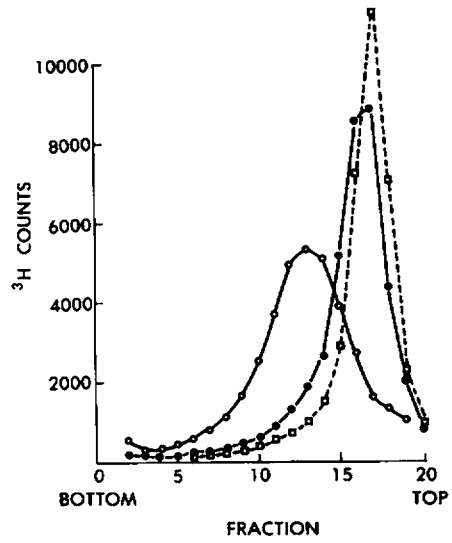

FIGURE 2

FIGURE 1 Pool equilibration rates. Cells were labeled overnight with [^{14}C]TdR and then pulse labeled with [^{3}H]TdR (50 μCi/ml, 57 Ci/mM), BrdUrd (10^{-5} M), and FUrd (10^{-6} M) for various lengths of time. The cells were then fixed and counted, and ^{3}H/^{14}C ratios were calculated and plotted as a function of the pulse label time. The term b/b_0 at time T is defined as ^{3}H cpm/^{14}C cpm at time T (min) divided by the slope of the linear portion of the curve times T (min). This number is a measure of the extent of substitution of endogenous thymine by exogenous thymines at time T in the labeled DNA (Povirk and Painter, 1976*a*).
FIGURE 2 A set of sucrose gradients from pulse-labeled E-11 cells that have been exposed to various fluences of 313 nm light, lysed on a 5–20% alkaline sucrose gradient, and centrifuged. Cells were exposed to 0 (○), 60 (●), or 180 s (□) of 313 nm light. Values for Q are calculated from such gradients.

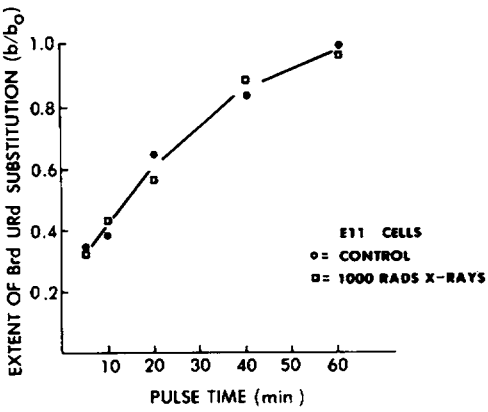

FIGURE 3

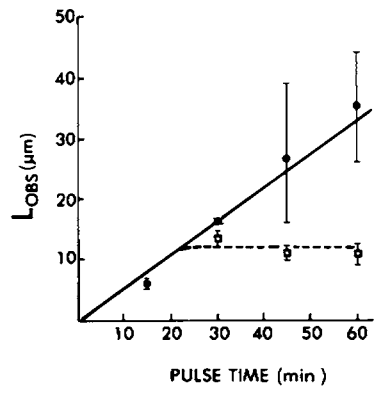

FIGURE 4

FIGURE 3 Extent of BrdUrd substitution into labeled DNA (b/b_0) as a function of time for E-11 cells in control (•) and X-irradiated cultures (□). ^{14}C-labeled cells were exposed to [^{3}H]TdR, BrdUrd, and FUrd as described in the legend for Fig. 1. b/b_0 was calculated for each point and is shown as the ordinate; time after beginning of the pulse label is shown on the abscissa. Irradiated cultures were exposed to 1,000 rads of X-rays immediately before pulse labeling.

FIGURE 4 L_{obs} (μM) as a function of duration of labeling time for E-11 cells in control and X-irradiated cultures. Cells were pulse labeled for lengths of time up to 60 min, and the length, L_{obs}, of the labeled piece of DNA was calculated as described in Methods. L_{obs} is presented for control (•) and X-irradiated (□) cultures. Standard errors are shown by error bars and increase with longer label times in controls, but not in X-irradiated cells. Presumably this reflects the fact that in irradiated cells, L_{obs} has stopped increasing and the distribution of sizes (at this value of L_{obs}) is the same from 25 to 60 min. In controls, L_{obs} continues to increase and as L_{obs} becomes larger, the DNA approaches the bottom of the gradient and the distribution of sizes also becomes broader, leading to increasing standard errors with longer labeling times.

Before this technique could be used for X-irradiated cells, we needed to know whether X-rays had any effect on pool equilibration times and degree of BrdUrd substitution into the pulse-labeled segment of DNA. To test this, ^{14}C-prelabeled cells were exposed to 0 or 1,000 rads of X-rays and pulse labeled for various times with [^{3}H]TdR plus BrdUrd, and the ^{3}H/^{14}C ratios were measured as described above. X-irradiation had no effect on the extent of BrdUrd substitution into DNA (b/b_0) when compared to control (0 rad) cultures (Fig. 3).

When HeLa or E-11 cells were incubated for up to 1 h with [^{3}H]TdR, BrdUrd, and FUrd, L_{obs}, appeared to increase continuously for up to 60 min (Figs. 4 and 5). If HeLa or E-11 cells were exposed to X-rays just before the incubation with [^{3}H]TdR, BrdUrd, and FUrd began, L_{obs} continued to increase almost linearly for approximately 25 min after the X-irradiation (during this time the graph of L_{obs} vs. time is coincident with that of the control cultures; Figs. 4 and 5). After 25 min, however, L_{obs} for irradiated cells leveled off. For E-11 cells this plateau value of L_{obs} for X-irradiated cells was about 12 μM, where it remained up to 60 min after the X-irradiation, whereas in nonirradiated cultures L_{obs} reached 35 μM at 60 min (Fig. 4). Similarly, L_{obs} for the

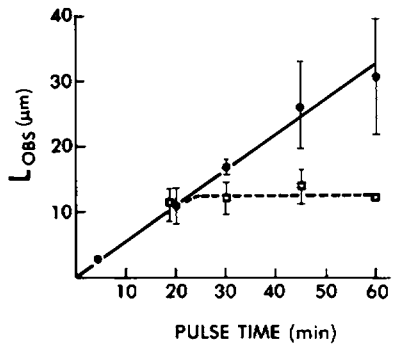

FIGURE 5

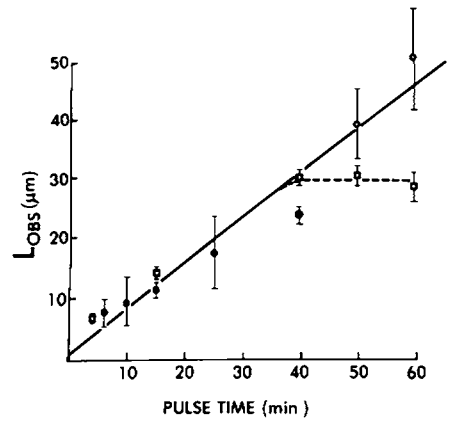

FIGURE 6

FIGURE 5 L_{obs} (μM) as a function of duration of labeling time for HeLa in control (•) and X-irradiated (□) cultures. Standard errors are shown by bars. Details are the same as described in the legend for Fig. 4.

FIGURE 6 L_{obs} (μM) as a function of duration of labeling time for CHO cells in control (•) and X-irradiated (o) cells. Details are the same as described in the legend for Fig. 4.

control HeLa cultures increased linearly and reached a value of approximately 30 μM at 60 min, but with irradiated HeLa cultures, L_{obs} reached a maximum, also at a value of about 12 μM, at 20 min (Fig. 5).

CHO cells showed a different response to X-rays compared to the two human cell lines (Fig. 6). The irradiated and control values remained coincident for about 40 min, after which time the values for L_{obs} of irradiated cultures remained constant at about 30 μM.

The estimate for average replicon size is actually about $\sqrt{2}$ times the plateau value of L_{obs} (see Appendix). Thus, the estimates for average replicon size are about 17 μM (= 1.4 × 12 μM) in human diploid and HeLa cells and about 42 μM (= 1.4 × 30 μM) in CHO cells.

The average rates of chain elongation were also estimated from these data by using the values of L_{obs} obtained from short pulse label times (Povirk and Painter, 1976*a*) and dividing them by the duration of the labeling time. These values were 0.5 μM/min for E-11, 0.65 μM/min for HeLa, and 1.1 μM/min for CHO.

DISCUSSION

If the length of a piece of growing DNA, L_{obs}, is measured as a function of time, it would be expected to increase continuously during a 1-h incubation time with labeled DNA precursors. This was found to occur in control cultures during the 1-h labeling period. After X-irradiation of human cells, however, L_{obs} did not increase after 20–25 min and remained constant for up to 1 h. This plateau value of L_{obs} is some function of the average replicon size in the replicating cells.

This interpretation is based on previous findings that moderate doses of radiation inhibit initiation of replicons in mammalian cells, but not the elongation and joining processes in replicons that had already initiated synthesis before irradiation (Watanabe, 1974; Makino and Okada, 1975; Walters and Hildebrand, 1975; Painter and Young, 1975). This can be explained by the hypothesis that a single hit within a cluster of replicons (Hand, 1975; Hori and Lark, 1973) blocks the initiation of all the replicons in that cluster (Painter and Young, 1976; Povirk and Painter, 1976*b*). This hypothesis receives support from recent work on the structure of the eukaryotic chromosome, which shows that the chromosome is organized into several orders of supercoiling (Cook and Brazell, 1975; Benyajati and Worcel, 1976). In human (HeLa) cells, the unit of supercoiling contains about 10^9 daltons of DNA (Cook and Brazell, 1975), which is also the estimated target size for the cluster of replicons blocked by low doses of X-radiation (Makino and Okada, 1975; Painter and Young, 1976; Povirk and Painter, 1976*b*). A logical model for the action of X-rays, then, is that they cause single strand breaks in the supercoiled subunits of the eukaryotic chromosome, causing them to "relax," i.e., to lose their supercoiled conformation. Supercoiling is required for replication of col E1 and λ DNA (Gellert et al., 1976) and for initiation of R6K plasmid DNA in *E. coli* (Lovett et al., 1974). Supercoiling therefore may also be required for initiation of eukaryotic DNA, and if so, initiation of replication in irradiated cells would not occur until the single strand break has been repaired and the supercoiled subunit returned to its original conformation. Replicons in clusters that had initiated before the irradiation are unaffected (Watanabe, 1974), presumably because once initiation has occurred, the subunit is no longer sensitive to a single strand break in one of its component replicons (although that particular replicon's duplication might be at least temporarily halted).

For the first 1–1½ h after exposure to 1,000 rads, DNA replication in HeLa cells consists primarily of elongation of replicons that had initiated before irradiation (Painter and Young, 1976). It follows then that L_{obs} will increase only until these replicons terminate. Thus the final value for L_{obs} in irradiated cells will be a function of the average replicon size, because the largest value for L_{obs} will be very slightly smaller than the distance between two adjacent origins. This is true because the radiation blocks initiation and therefore the only replicons labeled with [^{3}H]TdR and BrdUrd thereafter will be ones that initiated before irradiation. Those that initiated in the moments just before irradiation will elongate for 1–2 min (time between irradiation and addition of label) before they become labeled.

Several important assumptions have been made in estimating the value of L_{obs} and in the final calculation of replicon size: (*a*) the lag in [^{3}H]TdR incorporation is due to pool equilibration; (*b*) the kinetics of pool equilibration for BrdUrd and thymidine are the same; and (*c*) the triangular distribution of replicons (Fig. 7) actually represents the distribution of labeled and unlabeled segments of one class of replicons functioning after the cells are exposed to 1,000 rads of X-rays.

The curves of L_{obs} vs. t (Figs. 4–6) are linear for unirradiated populations, and the

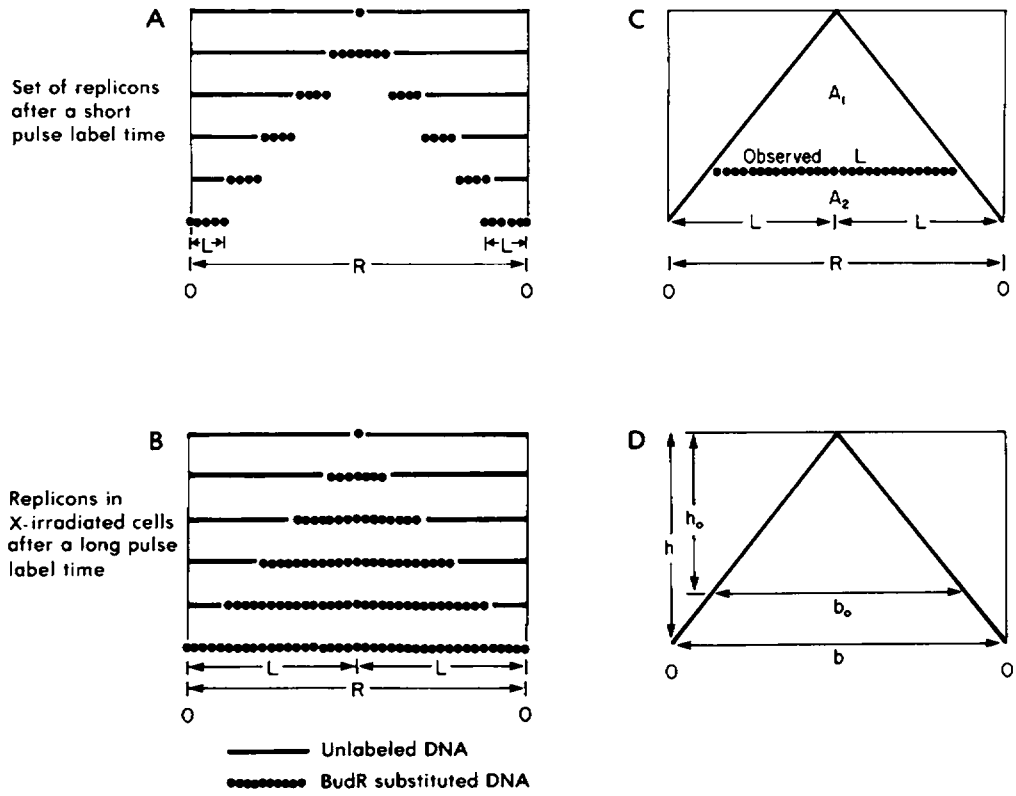

FIGURE 7 Illustration of triangular distribution of operating replicons. (A) Set of replicons showing distribution of BrdUrd-substituted DNA into operating replicons after a short pulse label. The letter O indicates the origin of a bidirectionally growing replicon. Note that, even here, there are some labeled segments longer than L and some shorter than L, because of replicon fusions during the pulse label. (B) Distribution of BrdUrd substitution in operating replicons after a long pulse label (i.e., after all replicons have fused). This distribution assumes that all initiation events are inhibited just before the beginning of the labeling period. (C) Redrawn triangular distribution from section B showing the observed L, measured experimentally. The maximum length of the labeled DNA piece is L and the replicon size R = 2L. The areas $A1$ and $A2$ are equal. (D) Redrawn from section C to illustrate the definitions of h, h_0, b, b_0 as used in the Appendix.

rate of chain elongation (= L_{obs}/pulse time) is constant with all pulse times for each cell line. The standard deviations of L_{obs} increase as L_{obs} increases because the standard deviation is always a constant percent of the mean molecular weight. Although the exact relationship of the plateau value of L_{obs} to replicon size in irradiated cells will have to be more exactly defined by future work, it is proportional to replicon size in a given cell line and can serve as a comparison between different cell types. It can also be used to measure changes in replicon size in a given cell line under different conditions, such as after exposure to mutagens or carcinogens or after transformation or viral infection.

The estimated value of about 17 μM for HeLa and E-11 replicons reported here is small compared with most values in the literature for mammalian cells (for reviews, see Edenberg and Huberman, 1975; Painter, 1976). The majority of these estimates came from methods using autoradiography, which lacks the resolution necessary to observe small replicons (Huberman and Riggs, 1968). The 42-μM value for CHO cells, however, is very similar to the estimates made by others for CHO cells in culture (Huberman and Riggs, 1968; Hori and Lark, 1973).

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APPENDIX

The average size of operating replicons must be some function of the plateau value of L_{obs} which was observed experimentally. To relate this plateau value of L_{obs} to an average replicon size, it is assumed that the operating replicons of a single-sized group of replicons form a triangular distribution as shown in Fig. 7. In addition it is also assumed that Fig. 7 shows the distribution of BrdUrd-substituted DNA after a short pulse label (Fig. 7 A) and after a long pulse label when all initiation events have been inhibited (Fig. 7 B).

Fig. 7 A shows a set of replicons, all the same size, which have been pulse labeled for a short time. If cells are exposed to X-rays to block new initiations, and allowed to incorporate label until all initiated replicons have terminated, the situation would be as shown in Fig. 7 B. The replicons almost finished replicating just before X-irradiation will fuse early (upper portion of the distribution), and L will be <1 replicon in length; at the bottom of the distribution, 1 replicon length (R) is equal to $2L$. The final plateau value of L_{obs} must be $<2L$ due to all of the early fusions of replicons in the upper part of the figure.

Because the method used to estimate L depends on 313-nm photolysis of BrdUrd, the target size of the observed L ($= L_{obs}$) will be such that the areas of the distributions of L representing L shorter than L_{obs} will be equal to the areas representing L longer than L_{obs}. This is shown in Fig. 7 C and relabeled in Fig. 7 D. When the areas above and below b_0 are equal,

$$\text{Total area} = A = \tfrac{1}{2}bh;$$

$$\text{area of upper triangle} = A_1 = \tfrac{1}{2}b_0h_0;$$

$$\text{and } A = 2A_1 = 2(\tfrac{1}{2}b_0h_0);$$

$$\text{and } bh = 2\,b_0h_0. \tag{1}$$

Because these are congruent triangles,

$$h/b = h_0/b_0 \text{ or } hb_0 = h_0b, \text{ and}$$

$$h = bh_0/b_0. \tag{2}$$

Substituting Eq. 2 into Eq. 1, $b(bh_0/b_0) = 2\,b_0h_0$; $b^2h_0 = 2\,b_0^2h_0$; $b^2 = sb_0^2$; $b = \sqrt{2}\,b_0$. Because $L_{obs} = b_0$, $R = 2L = b$, and $b = \sqrt{2}\,b_0$, then $R = \sqrt{2}\,b_0 = \sqrt{2}\,L_{obs}$.

This final distribution and conclusion that $R = \sqrt{2}\,L_{obs}$ depends on the assumptions that there is a triangular distribution of all replicon size groups and that there is an absolute

inhibition of new initiation events. This analysis does not apply to unirradiated cells where new initiations are always occurring.[1]

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[1] *Editor's Note:* Two of our four referees suggest that the proper relation is $L_{obs} = b_0/2$. Then $R = 2\sqrt{2}\, L_{obs}$. Rather than prolong the stalemate on this point, the authors' derivation has been accepted. The interested reader may settle the issue for herself or himself.