REPLICATING UNITS (REPLICONS) OF DNA IN CULTURED MAMMALIAN CELLS

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ABSTRACT Exponentially growing L5178Y mouse leukemic cells were incubated in the presence of 5′-bromodeoxyuridine (BUdR) for about 4 hr, transferred to the nonBUdR-containing medium for a certain period (t hours), and then pulse-labeled with TdR-^{3}H for 10 min. When DNA isolated from these cells was subjected to CsCl gradient centrifugation, the ^{3}H-activity was found to shift gradually from the heavy BUdR-containing peak to the light nonBUdR-containing peak with increasing time t. The average time required for the complete shift of ^{3}H-activity from the heavy to the light DNA fraction was 2.76 hr. Taking this as the average replicating time and the size of DNA fragments in the present preparation as 1.3×10^7 daltons, the rate of replication was found to be 2.1 nucleotides per strand per replicon per sec. By taking the upper limit of the average replicating time as the S period (7.3 hr), various characteristics of the replicating units, such as the lower and upper limits of average size, the average replicating time, the average number of replicating units, etc., were calculated (see Table I).

INTRODUCTION

In cells of higher organisms, Howard and Pelc (1953) and Lajtha, Oliver, and Ellis (1954) demonstrated that DNA synthesis occurs during the portion of interphase called the S stage. Taylor (1960), Hsu (1964), Stubblefield and Mueller (1962), Painter (1961), Peacock (1963), and Prescott and Bender (1963) have shown that DNA of different chromosomes is synthesized at different times within the S stage in a time pattern characteristic for each chromosome. Furthermore, DNA is synthesized simultaneously at multiple sites within each chromosome. Based on these observations and others, Taylor (1963) proposed a molecular model of chromosomes, in which the DNA of each chromosome is composed of many small replicating units joined together by 3′- and 5′-linkers. DuPraw (1965) proposed a similar, but slightly modified, model in which the replicating units are linked together end to end. Moses (1963) suggested a third model in which small circular units of DNA are linked through a backbone, probably composed of DNA and proteins. Plaut, Nash, and Fanning (1966) studied the replicating pattern of DNA in polytene chromosomes

of drosophila and suggested that the replicating units of DNA be called "replicons" after similar units in bacteria named by Jacob and Brenner (1963).

An attempt to characterize the replicating units of cultured mammalian cells (human carcinoma, HeLa) has been made recently by Painter, Jermany, and Rasmussen (1966). In the present paper, a method somewhat similar to that used by these authors is employed to estimate the rate of replication of DNA in mouse leukemic cells (L5178Y). From this rate, various characteristics of the replicating units have been determined. It was possible to compute absolute values for the average rate of replication per replicon and for the average number of replicons engaged in replication in *S* stage cells. Because of uncertainties regarding DNA fragmentation during isolation, the upper and lower limits for the following could be estimated: (*a*) average size, (*b*) average replicating time, and (*c*) average number of replicons per cell.

MATERIALS AND METHODS

Cell Line

The mouse leukemic cell line, L5178Y, kindly supplied by Dr. G. A. Fischer of Yale University and maintained in our laboratory for two years, was used in this study. The cells were cultured in Fischer's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% horse serum, 0.025% penicillin, and 0.005% streptomycin (Fischer and Sartorelli, 1964). The generation time of L5178Y cells, in our hands, was 10.8 ± 0.6 hr and the modal chromosome number, 41 (Watanabe and Okada, 1967). All studies were carried out on cell populations in the exponential growth phase.

Isolation of DNA from Cultured Cells

The cells, mass-cultured in tightly stoppered two-liter flasks, were centrifuged for 5 min at 2,000 rpm in a Servall Centrifuge (RC-2) (Ivan Sorvall, Inc., Norwalk, Conn.). The sedimented cells were then washed with isotonic saline, resuspended in 13 to 15 ml saline, and recentrifuged for 5 min at 2,000 rpm.

The washed, sedimented cells were suspended in saline-citrate (0.15 M NaCl-0.015 M Na citrate, pH 7) and homogenized in a Dounce's homogenizer (Blaessig Glass Specialties, Rochester, N.Y.) with a loose fitting plunger after addition of 0.1 volume of 5% sodium deoxycholate. DNA was isolated by means of a simplified version of the method of Marmur (1961). The homogenate was shaken with chloroform-isoamyl alcohol and centrifuged. The top layer of the centrifugate was overlayed by two volumes of ethyl alcohol, and the DNA was removed by winding around a glass rod. The isolated DNA was dissolved in saline citrate and treated repeatedly with chloroform-isoamyl alcohol until no more precipitate could be seen at the interphase. DNA was then isolated by alcohol precipitation. In most experiments, this fraction was used in CsCl gradient centrifugation runs. One of the main contaminants of DNA preparations, RNA, was sedimented to the bottom of CsCl gradient centrifugation tube and was easily separated from DNA. In some experiments, however, DNA was purified by treatment with ribonuclease (Marmur, 1961) followed by chloroform-isoamyl alcohol treatment and alcohol precipitation. This treatment degraded most of the RNA and left only DNA in the CsCl gradient pattern.

The amount of DNA isolated by the first step amounted to 40% of the total in the cells and the amount in the final step represented 10–20%. L5178Y cells, being lymphocytic in origin, contain rather small amounts of cytoplasm; mitochondrial DNA, if present, would have accounted for less than 1.5% of the total DNA (Schneider and Kuff, 1965; Nass, Nass, and Hennix, 1965; Corneo, Moore, Rao Sanadi, Grossman, and Marmur, 1966). It should be added that the sedimentation patterns were the same in the crude and enzyme-treated purified preparations.

BUdR (5'-bromodeoxyuridine) and TdR-^{3}H (thymidine-^{3}H) labeling

Cells in mass culture were incubated for 3–5 hr with BUdR ($5–8 \times 10^{-5}$ M) in the presence of TdR ($5–8 \times 10^{-6}$ M). (In preliminary experiments, the addition of thymidine was found to be necessary to keep the cells in the exponential growth phase for more than one generation.) After incubation, the cells were centrifuged at 1,000 rpm in a Servall Centrifuge (RC-2) for 5 min and then resuspended in fresh, pre-warmed medium. Fifteen minutes elapsed between the washing procedure and the next step, namely, incubation of cells for 10 min in fresh medium containing tritiated thymidine (10 μc/ml, 6.76 c/mM).

In later experiments, cell suspensions ($1–5 \times 10^5$ cells/ml) in the exponential growth phase were incubated with 10^{-4} M BUdR, 10^{-6} M CdR (deoxycytidine) and BUdR-^{14}C (0.02 μc/ml) for 3 hr. The cells were centrifuged, resuspended in fresh warm Fischer's medium, and divided into four fractions. One fraction was incubated immediately with thymidine-^{3}H (6.7 c/mM, 23 μc/ml) for 10 min. The other three fractions were incubated with fresh Fischer's medium in the presence of TdR (10^{-4} M) and CdR (10^{-6} M) for 1, 2, and 3 hr, respectively. The cells were then incubated for 10 min in 20 ml of fresh warm medium containing TdR-^{3}H (200 μc) and CdR (10^{-5} M). The sedimentation patterns in these experiments were similar to those of the early labeling experiments in which no thymidine chaser was used and the estimated replicating times in the two types of experiments were comparable.

CsCl Gradient Centrifugation

0.2 or 0.5 ml of DNA solution was added to 3.5 ml of CsCl solution (specific activity of 1.68 to 1.72) in each tube and the resultant suspension was centrifuged at 35,000 rpm for 65 hr in a Beckman Spinco L2 Centrifuge with SW 39 rotary head (Beckman Instruments, Inc., Palo Alto, Calif.). After centrifugation, the bottoms of the centrifuged tubes were punctured and 5–15 drops collected in each of 40–60 tubes. In every seventh tube, the density of the fraction was estimated by weighing 20-lambda micropipettes filled with the sample solution. The buoyant density of the BUdR-containing fraction was 1.75 ± 0.03 and that of the non-BUdR-containing fraction was 1.72 ± 0.03. The difference in the buoyant densities of the two fractions was 0.029 ± 0.004.

It should be pointed out that density measurement by use of a micropipette was simple but somewhat inaccurate. Thus, absolute densities were expected to have a large variation as is shown above. In contrast, the relative difference in densities between the two peaks was fairly accurate because the same pipette was used for each set of experiments.

After adding 1 ml of water, the optical density at 260 mμ of the aliquots was measured. Finally, 0.2 ml of the solution, 1 ml of water, and 10 ml of Bray's solution (1960) were placed in a glass bottle for assay of ^{3}H-activity in a Nuclear-Chicago liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

Sucrose Gradient Centrifugation

DNA solution (0.3 ml) was placed on top of 4 ml of a linear gradient of sucrose ranging from 20–5% sucrose in a solution containing 10^{-2} M Tris, 10^{-2} M KCl, 1.5×10^{-3} M $MgCl_2$ at pH

7.4, and centrifuged for 4 hr at 35,000 rpm in a Beckman Preparative L2 Centrifuge (Burgi and Hershey, 1963). After centrifugation, the bottom of the centrifuge tube was punctured, and 0.4 ml fractions were collected. To each fraction, 0.5 ml of water was added and the optical density at 260 mμ was read in a Zeiss spectrophotometer (Carl Zeiss Inc., New York). Then, 0.2 ml of each solution was used for counting ^{3}H-activity.

Determination of DNA

The amount of DNA was estimated by comparing the color of the diphenylamine reaction (Dische, 1955) with that of a standard set of calf thymus DNA solutions.

RESULTS

Estimation of One Unit of DNA per Cell

The value for the DNA content per cell is usually calculated by dividing the total amount of DNA in a cell suspension by the cell number. This gives only the average DNA content per cell whereas the actual DNA content of individual cells varies throughout the life cycle. Cells in G_1 stage (the postmitotic, pre-DNA synthesizing period) have one unit (2C) of DNA each. Upon entering the *S* stage (DNA synthesizing period), the cells begin to synthesize nucleic acid, and the DNA content increases progressively from one to two units. Upon completion of DNA synthesis, cells in G_2 stage (the post-DNA synthesizing, premitotic period) and in *M* stage (mitotic period) contain two units (4C) of DNA.

Stanners and Till (1960) derived an equation to convert the average DNA content per cell in an exponentially growing cell population to one unit (2C) of DNA. Using their equation and assuming that (*a*) the amount of DNA is linearly related to the time spent in *S* period (Stanners and Till, 1960), and (*b*) the dead cells (eosin-stained cells) (5–6% of the cell population) have one unit of DNA, the average DNA content per cell of our exponentially growing cell population was calculated to be 1.32 units of DNA. The observed amount of DNA per cell was $1.18 \pm 0.04 \times 10^{-11}$ g per cell; therefore, one unit (2C) of DNA found in one G_1 cell was 0.89×10^{-11} g per cell, and two units (4C) of DNA present in a single G_2 or *M* cell was 1.78×10^{-11} g per cell.

Relation of Size of DNA in our Preparations to the Size of Replicons

In the present experiments, it is extremely important to know the exact relationship between the size of the DNA in our preparation and that of the replicons. First, let us assume that each chromosome is made up of one or more replicating units of DNA. The DNA isolated from the cells may be composed of (*a*) a single replicon, (*b*) several replicons, or (*c*) fragments smaller than single replicons.

Life cycle analysis in our laboratory has shown that exponentially growing L5178Y cells divide every 10.8 hr (Watanabe and Okada, 1967). The *S* stage cells engaged in DNA synthesis constituted 62.4% of the total cell population and the *S* stage of individual cells lasted an average of 7.3 hr. When cells in the exponential growth

phase were allowed to grow for 4 hr, the cells which had just completed the S stage at the beginning of incubation passed through G_2 stage (1.3 hr), M stage (0.55 hr), and G_1 stage (1.7 hr). These cells reentered the S stage by the end of the incubation period. In other words, all cells (excluding dead cells) spent at least a part of the 4 hr incubation period in the S stage in which they synthesized a certain amount of

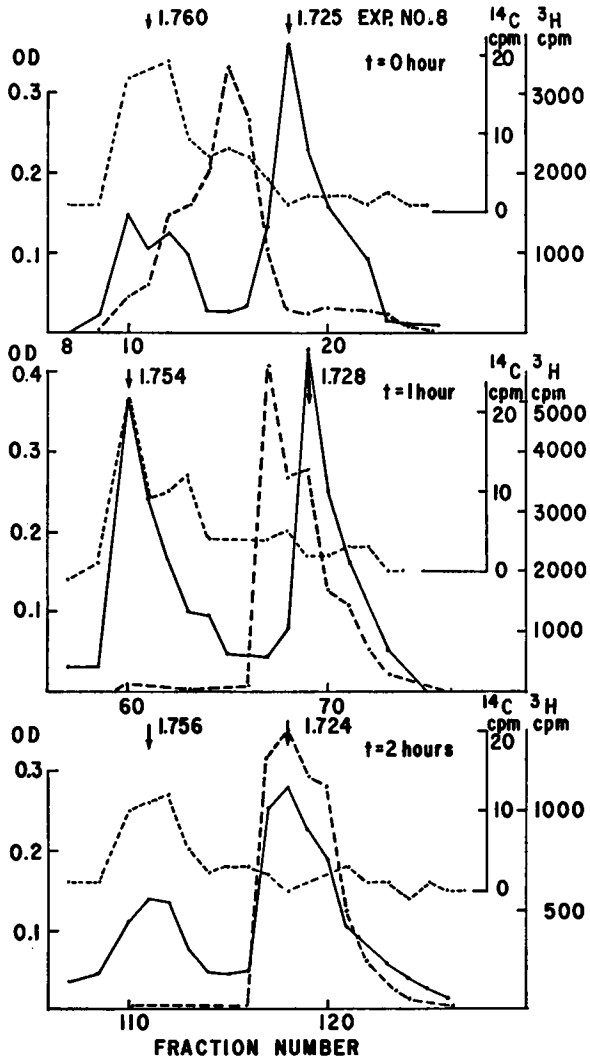

FIGURE 1 Sedimentation patterns of DNA isolated from cells. The cells were incubated with BUdR (10^{-4} M), BUdR-^{14}C (0.02 μc/ml), and CdR (10^{-6} M) for 3 hr, followed by the addition of cold thymidine (10^{-4} M) and CdR (10^{-6} M) for 0–2 hr, and incubated with TdR-^{3}H for 10 min. The solid line shows optical density at 260 mμ; thick, dotted line, ^{3}H-activity; and thin, dotted line, ^{14}C-activity.

DNA. When cells were grown in the presence of BUdR, the fraction of the DNA which replicated during the incubation period incorporated BUdR as well as thymidine and, thereby, became heavier than DNA which had not replicated during this period. Assuming that replicons replicated at a constant rate throughout the entire *S* period (which is not strictly correct), all DNA formed during the 4 hr incubation period would contain varying amounts of BUdR, provided that no significant fragmentation of DNA had occurred during the isolation procedure. The CsCl gradient centrifugal pattern, therefore, should consist of one peak, slightly heavier than the peak obtained from nonBUdR-containing DNA.

The observed CsCl sedimentation pattern, however, consisted of two peaks rather than one—a heavy peak containing BUdR and a light peak containing no BUdR (see Fig. 1). When the per cent of the total DNA in the heavy peak was estimated, the per cent of DNA in this peak formed per 1 hr incubation (7.6 ± 1.5%) was of the same magnitude as the theoretically calculated value (7.4%) for exponentially growing cells. This means that the heavy fraction consists almost entirely of BUdR-containing DNA rather than of a mixture of BUdR-containing and nonBUdR-containing DNA. This would seem to eliminate the possibility that the preparation was composed of DNA fragments made up of several replicons, i.e. BUdR-containing units attached to nonBUdR-containing DNA. Therefore, the present DNA preparation consisted of either (*a*) single replicons which replicate in a time period less than the whole *S* period and which can be separated into replicated BUdR-containing units and nonBUdR-containing units, or (*b*) fragments smaller than single replicons in which those portions containing BUdR can be separated from those not containing BUdR, or (*c*) both. In other words, the size of the DNA in our preparation represents the lower limit of the actual replicon.

Fractionation of DNA into Three Parts: (a) DNA not engaged in replication, (b) DNA which has just completed replication, and (c) DNA actively engaged in replication

Cells in the exponential growth phase were grown in the presence of BUdR for 3–4 hr, then, in the absence of BUdR for time t (t varies from 0–3 hr), and, finally, pulse-labeled with tritiated thymidine for 10 min. Immediately after pulse-labeling, the DNA was isolated and subjected to CsCl gradient centrifugation. The sedimentation pattern of such DNA preparations consisted of two peaks: one heavy, containing BUdR, and the other light, containing no BUdR (Figs. 1 and 2). At $t = 0$, the tritium activity was mostly associated with the heavy peak. With increasing time (t), the tritium-containing fraction shifted gradually from the heavy peak to the light peak, and after $t = 3$ hr, was found solely in the light peak.

The heavy BUdR-containing peak seen in Figs 1 and 2 consisted of DNA which must have been engaged in DNA replication during incubation with BUdR. The light peak, on the other hand, was composed of DNA which was *not* engaged in

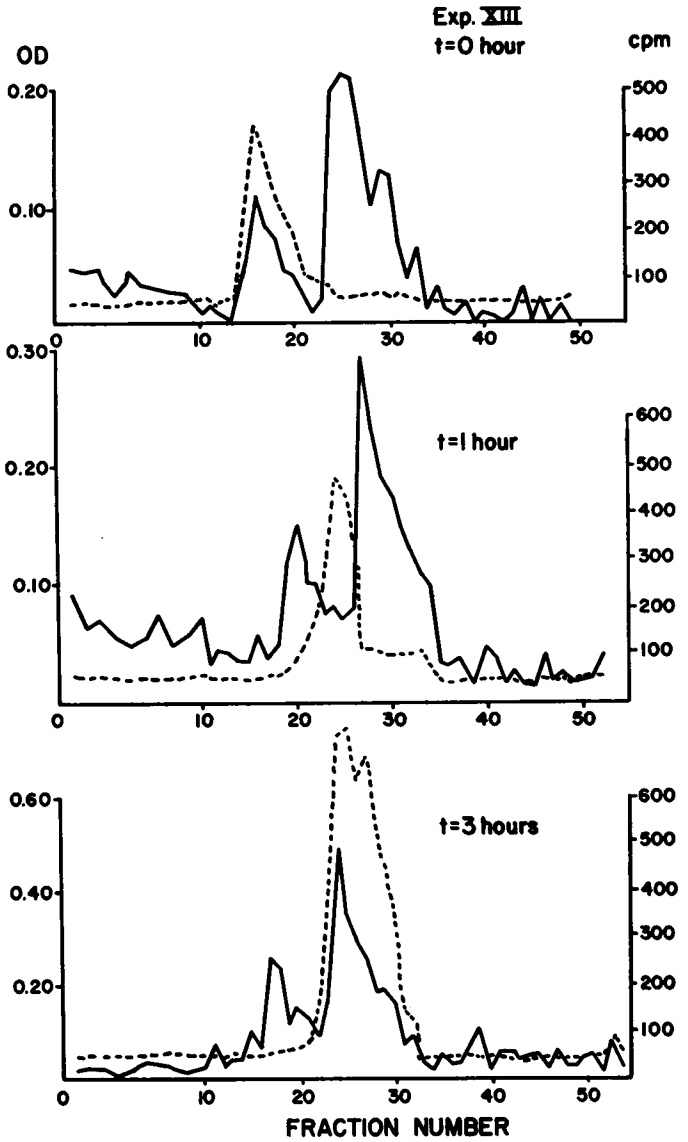

FIGURE 2 Sedimentation patterns of DNA isolated from cells sequentially incubated for 3.7 hr with BUdR (5×10^{-6} M) followed by incubation without BUdR for 0–3 hr. The cells were then incubated with TdR-^{3}H for 10 min. The solid line shows the optical density at 260 mμ; dotted line, the tritium activity in cpm.

replication during this incubation period. The fraction of DNA engaged in replication during pulse-labeling with thymidine-^{3}H (t = 0–3 hr) obviously contained tritium. At t = 0 hr, the tritium-containing fraction was found mostly in the heavy peak. Thus, the heavy peak at t = 0 consisted of the DNA fraction which had just completed replication and that which was still engaged in replication.

Estimation of the Minimum and Average Replicating Time of the Replicating Units

Figs. 1 and 2 show that the tritium activity shifted gradually from the heavy peak to the light peak with increasing time, t. At $t = 0$, the heavy peak containing most of the tritium activity was composed of DNA which had completed or was about to complete replication as well as that which had just started replication. Thus, the time required for tritium activity at this fraction to fall to zero corresponds to the time needed for DNA which has just started replication at $t = 0$ to complete replication. In other words, this is the average replicating time for this fraction of DNA.

In order to estimate the replicating time quantitatively, the percent of the total tritium activity in the heavy fraction was calculated and plotted against time, t (Fig. 3). In these experiments, cells incubated with BUdR were centrifuged and resuspended in fresh warm medium containing no BUdR. The minimum time required for this step was 15 min. The cells were then pulse-labeled with tritiated thymidine for 10 min. Because of the technical delay before pulse-labeling, the per cent of the tritium activity of the heavy fraction at $t = 0$ was always less than 100%. In estimating the replicating time, the linear portion of the per cent activity in the heavy fraction vs. time relationship was extrapolated to 100% and to 0% tritium activity, and the time between these two limits was designated the minimum average replicating time. This time turned out to be 2.76 hr $\pm$ 0.79 hr. Since this is the time required for replication of the lower limit of the replicons, one may conclude that the true average replicating time of the replicons lies between 2.76 hr and 7.3 hr (the entire S period).

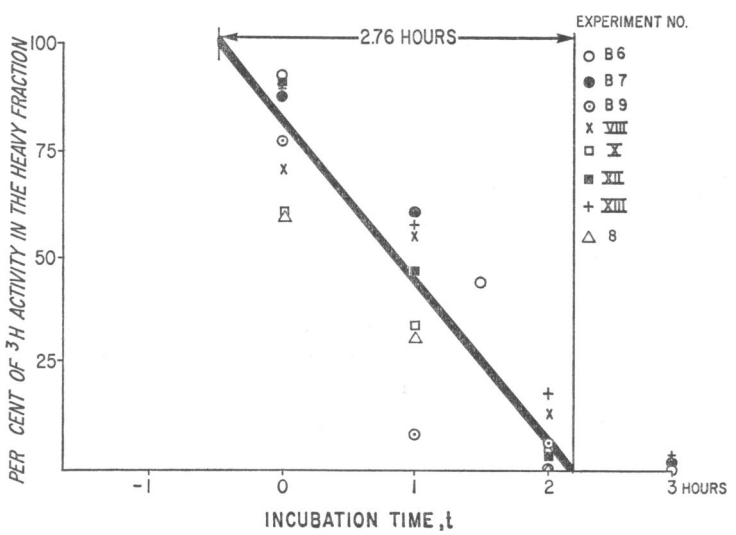

FIGURE 3 Relationship of per cent tritium activity in the heavy fraction with incubation time, t.

Estimation of the Minimum and Maximum Average Size of Replicating Units

In order to estimate the average size of the DNA in the present experiment, sedimentation constants ($s_{20,w}$) of two of our peparations were determined and found to be 26.3 and 27.4. The size in dalton units, therefore, calculated by the empirical formula of Eigner and Doty (1965) was 1.23×10^7 daltons and 1.36×10^7 daltons, respectively (average: 1.3×10^7 daltons).

In order to estimate the minimum average size of replicons, it was necessary to know whether the size of the heavy fraction of BUdR-containing DNA was similar to that of the DNA preparations estimated previously (1.3×10^7 daltons). After incubation of cells with BUdR and TdR-^{3}H for 3 hr, DNA was isolated. When such a DNA preparation was subjected to sucrose gradient centrifugation, the distribution of radioactivity was found to parallel nearly that of the optical density reading

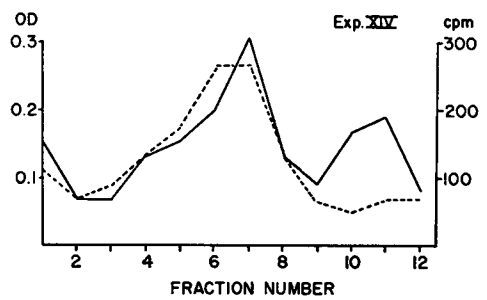

FIGURE 4 Sucrose gradient centrifugal pattern of DNA. DNA was isolated from the cells incubated with BUdR (10^{-5} M) and TdR-^{3}H (0.2 μc/ml) for 3.67 hr. The solid line represents the optical density at 260 mμ, and the dotted line, the tritium activity in cpm. In fraction no. 7 the ratio of optical density of 260 mμ vs. 280 mμ was 1.48, and that for optical density of 260 mμ vs. 240 mμ was 1.26. In fraction no. 11 the ratio of optical density of 260 mμ vs. 280 mμ was 1.28, and that for optical density of 260 mμ vs. 240 mμ was 0.88.

at 260 mμ (Fig. 4). This indicates that the size of the heavy fraction of DNA was essentially the same as that of the light DNA which constituted the bulk of our DNA preparation. Thus, the size of 1.3×10^7 daltons is regarded as the minimum average.

For the maximum average size, the following arguments were made: The time required for tritium activity to disappear from the heavy fraction is the time required for the DNA of this fraction to complete replication; also, the maximum length of time a cell can engage in DNA synthesis is 7.3 hr (the *S* period); therefore, the upper limit for the average size of the replicon is $1.3 \times 10^7 \times 7.3/2.76 = 3.4 \times 10^7$ daltons. Thus, the average size of actual replication would be somewhere between 1.3×10^7 and 3.4×10^7 daltons.

Average Rate of Replication per Replicon

The minimum replicating time of 2.76 ± 0.76 hr is the time required to replicate DNA with an average size of 1.3×10^7 daltons. Therefore, the amount of DNA

replicated per unit time per replicon was:

$$1.3 \times 10^7 \times \frac{1}{2.76 \times 60 \times 60} = 1308 \text{ daltons/sec/one replicon.}$$

If one accepts the semiconservative scheme of DNA replication (Djordjevic and Szybalski, 1960; Simon, 1961), the amount replicated by one DNA strand was 1310/2 = 654 daltons. Assuming the average size of one nucleotide unit of DNA to be 307 daltons, the number of nucleotides added to one strand per second was 654/307 = 2.1 nucleotide units/sec/strand.

Average Number of Replicating Units Engaged in Replication by Cells in S Stage

The average number of replicons engaged in replication in an *S* stage cell can be calculated assuming that (*a*) cells synthesized DNA at a constant rate throughout the *S* period, and (*b*) a constant number of replicons were engaged in replication throughout the *S* period. The total amount of DNA synthesized by an *S* stage cell was equal to one unit of DNA (0.89×10^{-11} g/cell), and the duration of the *S* period was 7.3 hr; therefore, the amount of DNA synthesized per second per cell was:

$$\frac{0.89 \times 10^{-11} \times 6.02 \times 10^{23}}{7.3 \times 60 \times 60} = 2.05 \times 10^8 \text{ daltons/sec/}S\text{ stage cell,}$$

or

$$\frac{2.05 \times 10^8}{307} = 6.68 \times 10^5 \text{ nucleotide units/sec/}S\text{ stage cell.}$$

The average number of replicons engaged in replication per *S* stage cell can be obtained by dividing this value by the rate of replication calculated in the previous section. Thus, $\frac{2.05 \times 10^8}{1308} = 1.6 \times 10^5$ replicons/sec/*S* stage cell.

Estimation of the Maximum and Minimum Numbers of Replicons per Cell

A G_1 cell contains one unit of DNA while G_2 and *M* cells contain two units of DNA. In other words, G_2 and *M* cells contain twice as many replicons as G_1 cells. In the following calculations, we consider only the total number of replicons of G_1 cells.

From the minimum size of replicons, one can calculate the maximum number of the replicons per one G_1 cell:

$$\frac{0.89 \times 10^{-11} \times 6.02 \times 10^{23} \text{ daltons/cell}}{1.3 \times 10^7 \text{ daltons/one replicon}} = 4.1 \times 10^5 \text{ replicons/cell.}$$

From the maximum size of the replicon, the minimum number of replicons per one G_1 cell was:

$$\frac{0.89 \times 10^{-11} \times 6.02 \times 10^{23} \text{ daltons/cell}}{3.4 \times 10^{7} \text{ daltons/one replicon}} = 1.6 \times 10^{5} \text{ replicons/cell.}$$

The actual number of replicons per one G_1 cell, then, was between 1.6 and 4.1 $\times$ 10^5 replicons per cell.

Since the average number of chromosomes of L5178Y cells is 41, the number of replicons per chromosome can be estimated by dividing the total number of replicons by the number of chromosomes. The maximum number of replicons per cell was $\frac{4.1 \times 10^5}{41} = 1 \times 10^4$ and the minimum number, $\frac{1.6 \times 10^5}{41} = 4 \times 10^3$. Since the length and DNA content varies from one chromosome to another, the calculated values are only rough estimates which give some idea as to the number of replicons per chromosome.

DISCUSSION

The evidence for the existence of small replicons of DNA in mammalian cells is fivefold: First, there are radioautographic studies of metaphase chromosomes of pulse-labeled cells in which multiple grain clusters in each chromosome suggest multiple sites of replication of DNA (Taylor, 1960; Hsu, 1964; Stubblefield and Mueller, 1962; Painter, 1961; Prescott and Bender, 1963); second, DNA synthesis in some chromosomes (e.g. *X* chromosome) (Taylor, 1960; Hsu, 1964) takes place only during a fraction of *S* period showing that the replicating time of some chromosomal DNA is definitely less than the *S* period; third, electron microscopic studies show small circular DNA-containing structures in pig sperm and wheat germ of approximately the same size as our replicons (Hotta and Bassel, 1965); fourth, radioautographic studies of DNA in HeLa cells (Cairns, 1966) suggest the presence of more than 100 replicating units per cell; and, fifth, by means of the end-to-end association of tritium and ^{14}C-BUdR labels in DNA, the number of replicating units in HeLa cells has been estimated to be 10^3–10^4 (Painter, Jermany, and Rasmussen, 1966), a somewhat smaller number than that in the present study.

Throughout the present analysis, only average values for replicons were estimated. In reality, the cell population in the exponential growth phase has inherent variations of its own as indicated by the following: (*a*) The duration of the *S* period varies from one cell to another (Sisken and Morasca, 1965; Killander and Zetterberg, 1965), and (*b*) the rate of DNA synthesis is not constant throughout the *S* period (e.g. Dendy and Cleaver, 1964; Terasima and Tolmach, 1963). In general, the rate of DNA synthesis in the early and late *S* stage is said to be slower than the rate in the middle *S* stage. However, whether the change in the rate of DNA synthesis results from variation in the number of replicons engaged in synthesis or from the variation in the rate of replication of individual replicons is not known. (*c*) The size of replicating units seems to vary according to the electron microscopic observations (Hotta and Bassel, 1965), and (*d*) the configuration of DNA in the middle of replication containing a

"growth point" is likely to be different from that before and after replication (Bonhoeffer and Gierer, 1963; Hanawalt and Ray, 1964). When discussing average values of replicons, one must be aware of these inherent variations.

The characteristics of the replicons estimated in the present experiment are summarized in Table I. From electron micrographic studies of interphase nuclei of amoeba, Taylor (1963) estimated the size of replicons to be 6.8×10^6 daltons. Hotta and Bassel (1965), on the other hand, examined electron photomicrographs of DNA isolated from pig sperm and wheat germ and found circular DNA units which varied in length from 6×10^6 daltons to 3×10^7 daltons. These values are in good agreement with the size of the replicons calculated in the present experiment.

TABLE I

CHARACTERISTICS OF REPLICONS IN L5178Y CELL

Number of replicons	
The average number of replicons per G_1 cell.	$1.6 \times 10^5 \leq$ actual number $< 4.1 \times 10^5$.
The average number of replicons engaged in replication in the S stage cell = 1.6×10^5 replicons/one S stage cell. The per cent of replicons engaged in replication in the S stage cell:	100% $\geq$ actual percent $>$ 37%.
The average number of replicons per one chromosome.	$4 \times 10^3 \leq$ actual number $< 1 \times 10^4$. The average number of replicons engaged in replication per one chromosome of one S stage cell = 4×10^3.
Average size of one replicon	
	1.3×10^7 daltons $\leq$ actual size $< 3.4 \times 10^7$ daltons
Average replicating time of one replicon	
	2.76 hr $\leq$ actual time $<$ 7.3 hr
Average rate of replication	
	4.2 nucleotide units/sec/one replicon
	2.1 nucleotide units/sec/one strand of one replicon
	6.80×10^5 nucleotide units/sec/one S stage cell

However, it should be pointed out that the electron microscopic study of chromatin in sea urchin sperm by Solari (1965) indicated that DNA molecules were larger than 1.72×10^8 daltons. Also, the radioautographic study of Chinese hamster DNA by Huberman and Riggs (1966) showed very long DNA molecules measuring $3.2–3.6 \times 10^9$ daltons. Although these long DNA molecules could be interpreted as many (perhaps 10 or more) linearly linked replicons, further investigation is necessary to find the relationship of these long DNA molecules to replicons.

Painter, Jermany, and Rasmussen (1966) using a somewhat similar approach to the present one, obtained the upper limit of 10^{10} daltons which is quite different from the upper limit of 3.4×10^7 daltons estimated in this paper. In the present experiments, however, average values are obtained while, in the other, the values estimated from one point in time might be biased in favor of longer replication units. The present studies take into consideration the life cycle of the cells. Also, it must be remembered that there are distinct differences between L5178Y and HeLa cell line (ploidy, generation time, etc.).

From his radioautographic study in HeLa cells, Cairns (1966) estimated the rate of replication of DNA in mammalian cells to be 0.5 μ per min. Assuming the average length of two nucleotide units to be 3.4 A (Watson and Crick, 1953), the rate of replication per DNA molecule can be expressed as 1470 nucleotide units per min or 25 nucleotide units per sec. Since both strands of bacterial chromosomes (Cairns, 1963) "replicate" at the same time, the rate of replication of each strand would be 12.5 nucleotide units per sec per strand. The average value of the rate in the present experiment is 2.1 nucleotide units per sec per replicon in one strand. Cairns' study may include only long fibers, whereas the present study deals with only average values. Therefore, the rates observed in these two studies could be considered to be in agreement.

As to the "absolute" replicating time, one could speculate from the variation of the rate of DNA synthesis during the *S* period assuming that the pattern of initiation of DNA synthesis is similar to that of the end of synthesis. In L cells and HeLa cells (Dendy and Cleaver, 1964; Terasima and Tolmach, 1963), the rate of DNA synthesis was maximal in the middle of the *S* period. This could mean that the "absolute replicating time" is of the order of half of the *S* period. In L5178Y cells, however, the rate of DNA synthesis decreased in the middle of *S* period (Watanabe and Okada, in press), suggesting that the absolute replicating time may be slightly less than half of the *S* period. This would place the absolute value to be in the range of 3–4 hr. Further studies are definitely needed to confirm this value.

The similarity of replicating units in bacteria and in the cells of higher organisms has been pointed out by Jacob, Ryter, and Cuzin (1966) as well as by Plaut, Nash, and Fanning (1966). The size of replicons estimated in the present paper is comparable to the amount of DNA in mitochondria of mammalian tissues (3×10^7 daltons per mitochondria and 14 units of 8.7×10^6 daltons) (Schneider and Kuff, 1965; Nass, Nass, and Hennix, 1965; Corneo, Moore, Rao Sanadi, Grossman, and Marmur, 1966) and to the amount of DNA per band per chromatid of polytene chromosome (3×10^7 daltons) (Plaut, Nash, and Fanning, 1966); this suggests that mitochondrial DNA and DNA in the polytene chromatids may be analogous to or, possibly, identical with replicons.

Finally, various properties of the replicating units of mammalian cells can be compared with those calculated by Pollard (1965) for *E. coli* (Table II). Although the rate of replicating per replicating units of mammalian cells is much slower than that of bacteria, mammalian cells have many more small units replicating simultaneously; therefore, the rate of total DNA replication per mammalian cell is greater than that per bacterial cell. It may be speculated that, in the course of evolution, higher organisms, such as mammalian cells, may have subdivided large DNA molecules into many small units to allow a more efficient and faster replication together with an efficient rapid transcription of the limited number of active replicating units (Littau, Allfrey, Frenster, and Mirsky, 1964; Paul and Gilmour, 1966).

TABLE II

COMPARISON OF CHARACTERISTICS OF REPLICONS OF DIFFERENT ORGANISMS

Characteristics of replicating unit	Organism		
	T2 phage*	*E. coli*‡	L5178Y
Number of replicons	1	1, 2, or 3	$1.6–4.1 \times 10^5$
Size of replicon (daltons)	1.3×10^8	2×10^9	$1.3–3.4 \times 10^7$
Rate of replication per organism (nucleotide units/sec/organism)	—	3000	6.8×10^5
Rate of replication per one replicon (nucleotide units/sec/one replicon)	—	1000–3000	4.2

* Hershey and Burgi, 1960.

‡ Pollard, 1965.

APPENDIX

Problems in Labeling of DNA with BUdR and TdR-^{3}H in Cultured Mammalian Cells

Several problems have been encountered in developing the labeling procedures: They are (*a*) the toxic effects of high concentration of thymidine and bromodeoxyuridine (over 10^{-4} M). This has been minimized by the addition of deoxycytidine (Morris and Fischer, 1963; Morris, Reichard and Fischer, 1963; Doida and Okada, 1967). (*b*) The toxic effects of incorporated ^{3}H. This has been shown to be minimal when ^{3}H-activity of less than 2 μc/ml was used (Burki and Okada, unpublished data). (*c*) Sufficient replacement of TdR incorporation by BUdR to insure a clear separation of heavy peak from light peaks. The intracellular thymidine pool size was computed to be about 10^{-4} eq (Burki and Okada, unpublished data). A high concentration of BUdR, over 3×10^{-5} M, is necessary to obtain a heavy peak separable from the light peak; and the thymidine or bromodeoxyuridine of the exogeneous origin remaining in the intracellular pool can be rapidly diluted by the high concentration of the thymidine intracellularly synthesized as a pool when the cells are washed out of exogeneous deoxyribonucleosides. This is quite clear in two types of experiments (e.g. Figs. 1 and 2) where, with or without cold thymidine chaser, the results obtained were the same.

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