subsequent generations producing a stable phenotypic but not genotypic change in the cell population. Further experiments are underway to identify the nature and function of the newly synthesized chromosomal proteins which have been shown here to remain associated with the DNA strand synthesized in the same cell cycle.

Acknowledgments

We thank Dr. Robert DeMars for helpful discussions and Ellen Roecker and Natalie Borenstein for expert technical assistance.

References

Anderson, L. E., and McClure, W. O. (1973), *Anal. Biochem. 51,* 173.
Bray, G. A. (1960), *Anal. Biochem. 1,* 279.
Gordon, J. S., Bell, G., Martinson, H. C., and Rutter, W. J. (1976), *Biochemistry 15,* 4778.
Ham, R. G. (1963), *Exp. Cell Res. 29,* 515.
Hancock, R. (1969), *J. Mol. Biol. 40,* 457.
Jackson, V., Granner, D., and Chalkley, R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4440.
Laemmli, U. (1970), *Nature (London) 227,* 680.
Lin, S. J., Lin, D., and Riggs, A. D. (1976), *Nucleic Acids Res. 3,* 2183.
Noll, M., Thomas, J. O., and Kornberg, R. D. (1975), *Science 187,* 1203.
Panyim, S., and Chalkley, R. (1969), *Biochemistry 8,* 3972.
Prescott, D. M., and Bender, M. A. (1963), *J. Cell Comp. Physiol. (Suppl. 1) 62,* 175.
Russev, G., Anachkova, B., and Tsanev, R. (1975), *Eur. J. Biochem. 58,* 253.
Seale, R. L. (1975), *Biochem. Biophys. Res. Commun. 63,* 140.
Smithies, O. (1970), *Science 169,* 882.
Taichman, L., and Freedlender, E. F. (1976), *Biochemistry 15,* 447.
Tsanev, R., and Russev, G. (1974), *Eur. J. Biochem. 43,* 257.
Tsanev, R., and Sendov, B. (1971), *J. Theor. Biol. 30,* 337.
Weintraub, H. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 247.

Deoxyribonucleic Acid Chain Growth and Organization of Replicating Units in HeLa Cells†

Stephen R. Planck‡ and Gerald C. Mueller*

ABSTRACT: A method for studying DNA chain growth and chromosomal organization of replicons in HeLa cells has been developed. DNA replication is initiated with bromodeoxyuridine followed by pulse labeling of active replicons with [^{3}H]thymidine and growth of the chains for finite intervals in unlabeled thymidine. Photolysis of the bromodeoxyuridine-DNA leader with 313-nm light releases the newly replicated chains that are then analyzed by sedimentation in alkaline sucrose gradients. This method of analysis provides data on the rate of chain growth, the bidirectionality of replication, and the distribution of the active replicons at specific intervals in the S period. Applying this method to cells caused to synthesize DNA at a lowered temperature (27 °C) or with protein synthesis restricted by cycloheximide revealed that the immediate reduction in the rate of DNA replication in both instances was due to a decreased rate of chain growth without derangement of the overall process.

DNA replication in the nucleus of eukaryotic cells involves the temporally ordered replication of many subchromosomal DNA units (replicons) which vary from 20 to 70 μm in length (Cairns, 1966; Huberman and Riggs, 1968; Callan, 1972). The ordering of the initiation of DNA synthesis in specific replicons remains unexplained; however, active replicons tend to be located in clusters giving rise to distinctive labeling patterns in different chromosomes (Taylor, 1959; Stubblefield and Mueller, 1962; Huberman and Riggs, 1968; Hori and Lark, 1973; Hand, 1975a). When a particular replicon is activated, the synthesis of DNA usually begins in the middle of the unit and proceeds in both directions (Huberman and Riggs, 1968; Huberman and Tsai, 1973; Weintraub, 1972; Hand and Tamm, 1973). The chain growth from this point in the overall 5′ → 3′ direction appears to proceed by the discontinuous formation of 5-6S pieces (Okazaki fragments) that are ligated to form mature DNA (Schandl and Taylor, 1969; Nuzzo et al., 1970; Kidwell and Mueller, 1969; Goldstein and Rutman, 1973; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975). The opposing strand may proceed by the same mechanism or by direct extension (Hershey and Taylor, 1974; Friedman, 1974). The molecular events which regulate the initiation of specific segments, the chain growth rates, and ligation of adjacent replicons are as yet little understood.

The present study is directed to the development of a method for investigating DNA chain growth and the organization of replicating units during chromosomal replication both in living cells and in subcellular systems. The general protocol involves the initiation of DNA replication in synchronized cells with bromodeoxyuridine so as to introduce a photolabile DNA segment in each active replicon, a subsequent pulse labeling

† From the McArdle Laboratory for Cancer Research, The University of Wisconsin, Madison, Wisconsin 53706. *Received May 3, 1976.* This work was supported by grants from the National Cancer Institute (CA 07175, T01 5002). G.C.M. is a recipient of a Research Career Award from the United States Public Health Service.

‡ Present address: Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland 20014.

of each growing point with [^{3}H]thymidine, and the continuation of DNA replication with unlabeled thymidine for specified intervals. In this way, most segments of [^{3}H]DNA have a bromodeoxyuridine leader on one end and an unfinished chain of unlabeled DNA on the other end. Irradiation of this DNA with 313-nm light introduces single-stranded breaks in the BUdR-DNA[1] with no direct effect on the TdR-DNA (Regan et al., 1971b). Alkaline denaturation then releases the newly replicated DNA chains that extend from such BUdR-DNA segments and permits determination of their size by sedimentation in alkaline sucrose gradients. With this method, DNA chain growth has been studied in synchronized HeLa cells under conditions of normal and restricted protein synthesis and at normal and reduced temperature. In addition, measurements of chain maturation and the amount of clustering amid active replicons have been carried out. The present protocol extends the use of UV light to selectively cleave one segment of DNA from another. The principle has proved successful in the study of the bidirectional nature of DNA replication (Weintraub, 1972), DNA repair mechanisms (Regan et al., 1971a,b; Lehmann, 1972a), and the effect of caffeine on DNA replication (Lehmann, 1972b).

Materials and Methods

Cell Culture and Synchronization. HeLa cells were cultured in suspension using modified Eagle's medium as described previously (Mueller et al., 1962). To synchronize cells for S-phase studies, a thymidine-deficient state was induced by adding amethopterin (1 μM) and adenosine (50 μM) to cells growing logarithmically at a density of 2.0–2.5 $\times$ 10^5 cells/mL. Thymidine or bromodeoxyuridine was added 16 h later to reverse the thymidineless state and to enable cells that were collected in early S phase to engage in DNA synthesis (Mueller and Kajiwara, 1969).

Analysis of DNA Size. The molecular weight of single-stranded DNA was calculated from the data obtained by sedimentation through alkaline sucrose gradients. To avoid shearing the DNA, the cells were lysed and the DNA was denatured directly on top of the gradients (McGrath and Williams, 1966). In practice, 37-mL gradients were prepared in polyallomer tubes and allowed to sit overnight at 8 °C. The gradients contained 0.3 M NaOH, 0.7 M NaCl, 1 mM EDTA, 60 mM *p*-aminosalicylate, and 5–20% sucrose (Parkhurst et al., 1973). Just prior to use, 100 μL of the lysing solution (1 M NaOH–0.1 M EDTA) was layered on the gradients. Immediately, 2–4 $\times$ 10^5 cells in 50 μL of SSC (0.15 M NaCl–15 mM sodium citrate) were layered and allowed to lyse for at least 30 min at 8 °C prior to beginning sedimentation. The gradients were centrifuged in a Beckman SW 27 rotor at 8 °C usually to an $\omega^2 t$ of 2.37 $\times$ 10^{11} rad^2/s. The actual speeds and times for the individual experiments are given in the figure legends.

The gradients were fractionated by overlaying them with water and displacing the fractions through a hole in the bottom of the tube at a rate of 2.4 mL/min; thirty 33-drop fractions (about 1.3 mL) were collected. Fraction 29 contained the top of the gradients. Fifty micrograms of bovine serum albumin (fraction V) and 200 μg of DNA were added to each fraction to facilitate the recovery of fractionated material during precipitation. The DNA and protein were coprecipitated overnight with cold 8% Cl_3CCOOH. The acid-insoluble precipitate was collected on Whatman GF/C filter discs and washed with cold 2% Cl_3CCOOH and then 95% ethanol. The DNA was solubilized by incubating the filters overnight at 22 °C in 200 μL of Soluene 100 (Packard). Toluene–2,5-diphenyloxazole scintillation fluid was added and the samples were left at room temperature for at least 8 h to decrease the chemiluminescence before the radioactivity was measured in a liquid scintillation spectrometer.

Calibration of the alkaline sucrose gradients was accomplished by use of the following labeled bacteriophage DNA standards: ϕX174, 16 S; T7, 37 S; T4, 70 S. Molecular weights were calculated according to the relation derived by Studier (1965).

Assay for DNA Synthesis. DNA synthesis was measured by the incorporation of radioactive thymidine or bromodeoxyuridine into acid-insoluble material. The cells were harvested by centrifugation at 800*g* for 7 min and resuspended in SSC. An equal volume of cold 0.8 M $HClO_4$ was added and the precipitate washed twice by resuspension in cold 0.4 M $HClO_4$ containing 10 mM sodium pyrophosphate. The precipiate was dissolved in 0.2 mL of 88% HCOOH at room temperature and washed into scintillation vials with 0.3 mL of water. Six milliliters of Scintisol (Isolabs) was added and the radioactivity determined with a liquid scintillation spectrometer.

Conditions for Ultraviolet Irradiation. 2–4 $\times$ 10^5 cells in 50 μL of SSC were placed in microtiter plate (Linbro) wells 5 cm below a Westinghouse Model FS20 sunlamp bulb. In order to absorb lower wavelength UV light and heat from the bulb, the bottom half of a 15 $\times$ 60 mm Falcon plastic tissue culture dish containing a 1 cm deep layer of a thymidine solution (2 mg/mL) was positioned directly above the microtiter plate containing the samples. Under the conditions employed, the 313-nm band of the sunlamp constituted the only effective irradiation. The entire operation was carried out in a cold room at 4 °C and the samples irradiated for 2 h except as noted.

Results

Photolysis of BUdR-DNA. In the experiments which follow, UV light has been used to cleave photolytically BUdR-DNA segments from growing chains of thymidine containing DNA so that the latter can be accurately sized by alkaline sucrose gradients. This approach requires extensive fragmentation of the BUdR-DNA so that the amount of BUdR-DNA remaining attached to the newly replicated TdR-DNA chains is negligible; at the same time, the TdR-DNA must not be degraded. To establish the duration of irradiation necessary to achieve this end point, cells labeled with [^{3}H]bromodeoxyuridine and [^{14}C]thymidine were combined and irradiated for increasing times. It was found that irradiation of the cells for 2 h as described under Materials and Methods was sufficient to degrade the BUdR-DNA to pieces less than a few hundred nucleotides in length while the TdR-DNA remained unchanged. Under the same conditions, the TdR-DNA which was base paired to the template strand was cleaved once for every 10^3 to 10^4 breaks in the BuDr-DNA. Even with 60 min of irradiation, the BUdR-DNA fragments that remained attached to the labeled TdR-DNA chains were sufficiently small so as not to affect the sedimentation analysis significantly.

Analysis of DNA Chain Growth in Vivo. The selective destruction of BUdR-DNA by irradiation with ultraviolet light provides a convenient means for studying the DNA strand growth and maturation in vivo. In a typical experiment, DNA synthesis was initiated by the addition of bromodeoxyuridine (40 μg/10^8 cells; 0.1 μg/mL) to cells which had been synchronized with amethopterin for entry into S phase. The cells

[1] Abbreviations used: BUdR-DNA, DNA containing 5′-bromodeoxyuridine; TdR-DNA, DNA containing deoxythymidine; UV, ultraviolet; SSC, 0.15 M NaCl–15 mM sodium citrate.

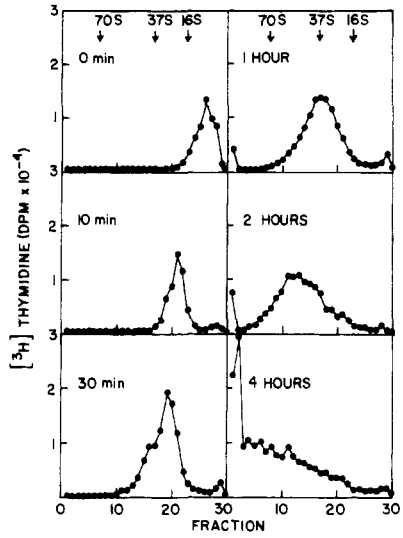

FIGURE 1: Elongation of the [^{3}H]TdR-DNA strand released from bromodeoxyuridine leaders. DNA synthesis was initiated in amethopterin-blocked cells by the addition of bromodeoxyuridine (40 μg/10^8 cells). After 2 h, the cells were pelleted, resuspended at 2 × 10^6 cells/mL in AA media (fresh media plus 1 μM amethopterin and 50 μM adenosine), and incubated for 30 min. The DNA growing points were then labeled with[^{3}H]thymidine (5 μCi/mL, 55 Ci/mmol) for 2 min, and the labeling was stopped by adding 10 volumes of cold SSC or by dilution of the [^{3}H]thymidine with an equal volume of warm AA media containing 5 μg/mL thymidine, in which case, synthesis was halted after the indicated intervals by adding cold SSC. To fragment the BUdR-DNA, the cells were harvested, resuspended in SSC (6–7 × 10^6 cells/mL), and irradiated for 90 min. The samples were then fractionated in alkaline sucrose gradients as described under Materials and Methods. Centrifugation was for 15 h at 20 000 rpm.

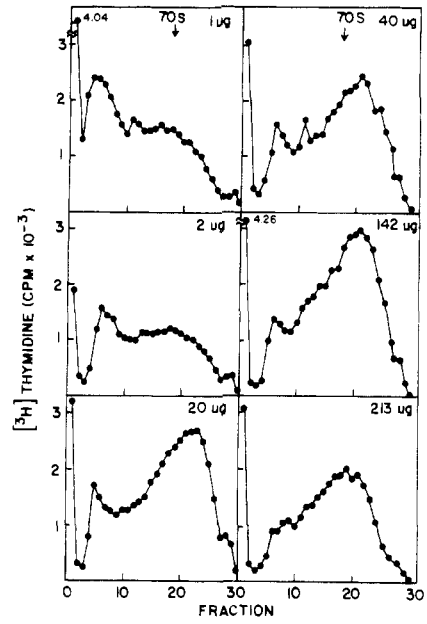

FIGURE 2: Distribution of active growing points at various times in S phase. DNA synthesis was initiated in amethopterin-blocked cells by addition of 1, 2, 20, 40, 142, or 213 μg of bromodeoxyuridine/10^8 cells. The cultures receiving 1, 2, 20, and 40 μg bromodeoxyuridine/10^8 cells were incubated for 1.8 h while the cultures receiving 142 and 213 μg of bromodeoxyuridine/10^8 cells were incubated for 5.8 and 7.8 h, respectively. The cells were then pelleted, resuspended in AA media (2 × 10^6/mL), and incubated for 30 min. To label the replicating DNA, a 1-mL aliquot of cells was incubated with 5 μCi of [^{3}H]thymidine for 2 min. The labeling was then stopped by adding 5.7 mL of cold AA media containing 2.94 μg/mL thymidine. To allow DNA replication to resume, the cells were pelleted and resuspended in 6.7 mL of warm AA media containing 2.5 μg/mL thymidine. The cultures that had initially received 1, 2, 20, and 40 μg of bromodeoxyuridine/10^8 cells were incubated for 12 h while the cultures that had received 142 and 213 μg of bromodeoxyuridine/10^8 cells were incubated for 8 and for 6 h, respectively. Forty milliliters of cold SSC was added to stop DNA synthesis and the cells were harvested, resuspended in SSC (6–7 × 10^6/mL), and irradiated for 2 h. Fifty-microliter aliquots were run in a alkaline sucrose gradients; centrifugation was for 6.2 h at 22 000 rpm.

were incubated for 2 h at 37 °C to incorporate this amount of added nucleoside into active replicons. The growing points were then pulse labeled for 2 min with [^{3}H]thymidine of high specific activity. Immediately thereafter excess unlabeled thymidine was added to dilute the remaining isotope and the incubation continued for the indicated interval to allow for DNA chain growth. With this protocol, the [^{3}H]thymidine pulse labels short sections of each replicating unit irrespective of its stage of development. Such units range from those that have just initiated synthesis to those that have completed synthesis and are in the process of ligation to adjacent replicons. In all cases, however, the [^{3}H]thymidine-labeled DNA extends from the BUdR-DNA that was introduced in the replicating sites at the time of the reversal of the thymidineless state.

Irradiation with UV light to cleave the BUdR-DNA leaders releases the growing DNA chains containing the [^{3}H]thymidine; this is attended by a shift of the isotope distribution in sucrose gradients to the regions of smaller chain lengths. Under these circumstances, the length of each newly replicated chain reflects the chain growth after the switch from bromodeoxyuridine to thymidine during the indicated intervals, the amount of ligation of adjacent completed replicons, and the distribution of the bromodeoxyuridine-labeled replicons in the region of the genome undergoing replication during the particular interval of the S phase which was studied. Since only a short section of each replicon was labeled, the amount of label that is contained in a given gradient fraction reflects primarily the number of chains of that size. Figure 1 depicts this chain growth over intervals up to 4 h. Calculating from the peak positions, the DNA chains grew at 1.5 to 4.3 × 10^5 daltons/min during the first hour of the chase interval. These rates are one-quarter to one-half the growth rates proposed by other laboratories (Hori and Lark, 1973; Hand and Tamm, 1973; Housman and Huberman, 1975; McFarlane and Callan, 1973; Weintraub and Holtzer, 1972; Gautschi et al., 1973). These data are in general agreement with those of Weintraub (1972) who first used photolytic cleavage of bromodeoxyuridine-prelabeled DNA to demonstrate bidirectional DNA replication at the start of S phase in chick erythroblasts. The present study indicates further that bidirectional DNA replication also holds for replicons which are operative in mid S phase.

Distribution of Active Growing Points in the Genome. Autoradiographic studies of metaphase chromosomes from pulse-labeled cells (Taylor, 1959; Stubblefield and Mueller, 1962) and density-labeling experiments in synchronized cells (Mueller and Kajiwara, 1966; Amaldi et al., 1969) show that DNA replication in a given S period is a highly ordered process. In addition, autoradiographic analysis of DNA fibers from cells pulse-labeled with [^{3}H]thymidine shows that replication proceeds simultaneously from multiple sites on the same DNA fiber and that the active units (i.e., replicons) are clustered along the chromosomal DNA (Huberman and Riggs, 1968; Hori and Lark, 1973; Hand, 1975a). To explore these observations using the present technology, DNA replication was initiated with bromodeoxyuridine and the growing points pulse labeled with [^{3}H]thymidine as described in the previous experiment; however, this time the cold thymidine chase was prolonged until the cells had completed DNA replication. After

TABLE I: Cycloheximide Effect on Thymidine Incorporation and DNA Chain Growth.

	Chase Interval (min)		
	10	30	60
	Mol wt ($\times 10^6$)[a]		
Control	4	11	20
Cycloheximide	4	8	14
% of control	100	73	70
	Thymidine incorp[b] (pmol/10^6 cells)		
Control	0.091	0.20	0.28
Cycloheximide	0.097	0.16	0.21
% of control	106	79	75

[a] From Figure 3. [b] From parallel cultures containing 2.5 μg/mL [^{3}H]thymidine (40 μCi/mg).

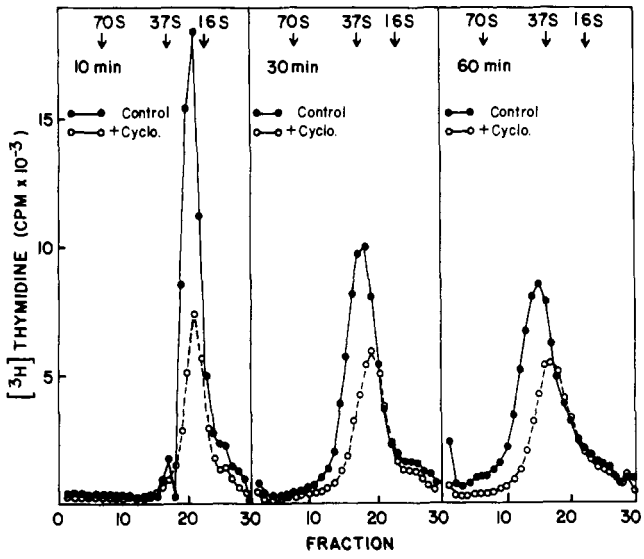

FIGURE 3: Effect of cycloheximide on DNA elongation. DNA synthesis was initiated in amethopterin-blocked cells by adding bromodeoxyuridine (40 μg/10^8 cells). After incubating for 1.8 h, the cells were pelleted, resuspended in AA media (2 $\times$ 10^6/mL), and incubated an additional 30 min. The DNA growing points were then labeled for 2 min with 20 μCi of [^{3}H]thymidine (55 Ci/mmol). DNA replication was continued after diluting the [^{3}H]thymidine by the addition of AA media (23 mL) containing thymidine (2.94 μg/mL). At each time point, a 6.7-mL aliquot was diluted with 40 mL of cold SSC to stop DNA synthesis. The cells were harvested, irradiated, and analyzed on alkaline sucrose gradients as described in Materials and Methods. Centrifugation was for 15 h at 20 000 rpm. (●—●) Control, (○- - -○) 200 μg/mL cycloheximide present during the thymidine labeling and chase.

irradiating to degrade the BUdR-DNA segments, the size of the labeled thymidine DNA chains should represent the size of the DNA segments between the bromodeoxyuridine-substituted regions that contained at least one active growing point during the [^{3}H]thymidine-labeling period. The smallest fragments would result from active replicons initiated with BUdR-DNA segments located adjacent to a replicon that had also initiated synthesis with bromodeoxyuridine. These fragments would be shorter than the length of one replicon and labeled at one or both ends. Larger fragments would result from the addition of late-replicating replicons to the DNA segments extending from the bromodeoxyuridine-substituted segments. One would also expect to see some fragments that had been labeled in the middle when new replicons were initiated during the [^{3}H]thymidine-labeling period itself; however, this fraction is most likely too small to make a significant contribution to the total picture.

The results of such an experiment are shown in Figure 2. DNA synthesis was initiated in amethopterin-blocked cells by adding graded amounts of bromodeoxyuridine (1, 2, 20, 40, 142, and 213 μg/10^8 cells) to produce nuclei with increasing fractions of their chromosomal DNA labilized with bromodeoxyuridine. Depending on the levels of bromodeoxyuridine added, the cells synthesized roughly 0.9, 1.3, 10, 16, 67, and 95% of their DNA with this analogue (data not shown) prior to the pulse with [^{3}H]thymidine and chase with unlabeled thymidine. A comparison of the distribution of radioactivity in fractions 10–30 from the gradients (Figure 2) reveals a striking similarity in the profiles of those samples having synthesized 10% or more of their DNA with bromodeoxyuridine. In general, 30–40% of the label was recovered in fragments of less than 60 $\times$ 10^6 daltons. Assuming a replicon size range of 30–60 $\times$ 10^6 daltons (Huberman and Riggs, 1968; Hori and Lark, 1973; Housman and Huberman, 1975; McFarlane and Callan, 1973; Hand, 1975a), these fragments must have been labeled by growing points in adjacent replicons or only separated by 1 unit. This argues for a significant clustering of the active replicons. Another 30–40% of the label sedimented in the 60–300 $\times$ 10^6 dalton range that corresponds to 5–10 replicons. The smaller fraction of labeled chains that are less than 300 $\times$ 10^6 daltons in the two samples at the beginning of S phase suggests that the first initiation sites are not clustered to the degree that was found for later initiating replicons.

The peak sedimenting in fractions 4–9 (150–160 S) has been seen under a wide variety of conditions by us and others (Parkhurst et al., 1973; Lett et al., 1970). While it may in fact reflect a subunit of chromatin as proposed by Lett et al. (1970), the present studies show it to be independent of the specific combination of bromodeoxyuridine and UV treatments used in these experiments and is therefore not considered further.

Effects of Protein Synthesis Inhibition on DNA Replication. Inhibitors of protein synthesis have been known for many years to interfere with the DNA replication process (Mueller et al., 1962). This interference has been attributed to decreased rates of DNA chain elongation (Gautschi and Kern, 1973; Gautschi, 1974; Hand and Tamm, 1972; Weintraub and Holtzer, 1972; Seale and Simpson, 1975), decreased frequency of initiation of new replication units (Hori and Lark, 1973; Hand and Tamm, 1972; Hand, 1975b), and impaired ligation of intermediate sized DNA fragments (Werner and Maier, 1975; Hyodo et al., 1971; Seale and Simpson, 1975).

To study the dependence of DNA chain growth on protein synthesis, synchronized cells were incubated with 200 μg/mL cycloheximide which rapidly stops protein synthesis and after a 10-min lag reduces the rate of DNA synthesis (Table I). This delay in the inhibition of DNA synthesis appears to be a consequence of the synchronization and reversal procedure since it was not observed with logarithmically growing cells. Weintraub and Holtzer (1972) reported a similar observation with 5′-fluorodeoxyuridine-treated chicken cells. If the cycloheximide is removed during the first hour, both protein and DNA syntheses quickly resume; however, this reversibility decreases with time of cycloheximide treatment.

To determine if decreased strand elongation can account for the reduced rate of DNA synthesis, bromodeoxyuridine-prelabeled cells were pulse-labeled with [^{3}H]thymidine, chased with unlabeled thymidine, and irradiated. The DNA was then fractionated on alkaline sucrose gradients (Figure 3). For each

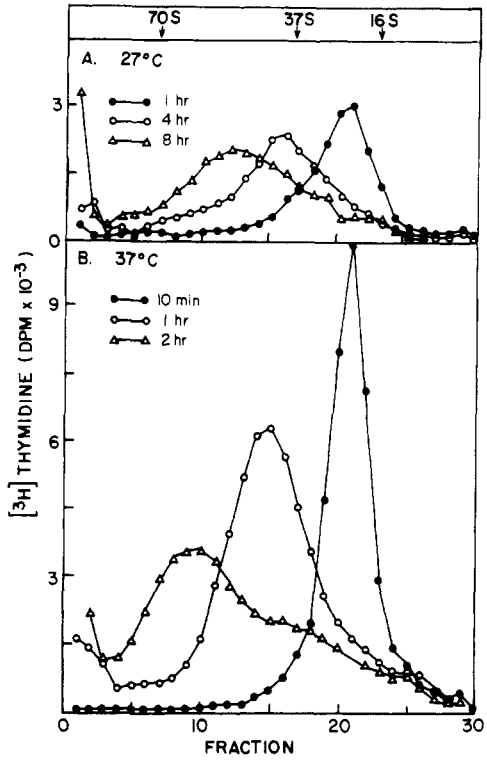

FIGURE 4: DNA elongation at 27 and 37 °C. DNA synthesis was initiated in amethopterin-blocked cells by adding bromodeoxyuridine (40 μg/10^8 cells) and incubating for 1.8 h. The cells were then pelleted and resuspended in AA media. After the medium change, half of the culture was incubated at 27 °C for 45 min and half incubated at 37 °C. The DNA growing points in 8×10^6 cells (4 mL) were then labeled for 2 min with [^{3}H]thymidine (5 μCi/mL, 55 Ci/mmol). Excess [^{3}H]thymidine was removed by adding 23 mL of cold AA media containing 2.94 μg/mL thymidine, centrifuging down the cells, and resuspending them in 27 mL of AA media containing 2.5 μg/mL thymidine at either 27 or 37 °C. DNA replication was continued for the indicated intervals and then the cells were harvested, irradiated, and analyzed with alkaline sucrose gradients as described. Centrifugation was for 9.6 h at 25 000 rpm. (A) At 27 °C, chase time: (●—●) 1 h; (○—○) 4 h; (△—△) 8 h. (B) At 37 °C, chase time: (●—●) 10 min; (○—○) 1 h; (△—△) 2 h.

chase interval, the [^{3}H]DNA from the cychoheximide-treated cells sedimented slower than that from the control cells; however, there was no accumulation of small fragments as Werner and Maier (1975) have reported. Furthermore, the rates of [^{3}H]thymidine incorporation and chain growth were changed proportionally during cycloheximide treatment of parallel cultures (Table I). Therefore, the acute depression of DNA synthesis by cycloheximide must involve a process which results in primarily a reduced rate of strand growth without producing a striking derangement of the overall process of DNA synthesis. Similar results were obtained using 25 μg/mL puromycin instead of cycloheximide, suggesting that inhibition of protein synthesis is the critical factor rather than some unknown toxicity of cycloheximide in the system.

During the course of this work, we observed that prolonging the treatment of the cells with high doses of cycloheximide or puromycin beyond 1 h led to degradation of the cellular DNA. The DNA, however, was not degraded to acid-soluble pieces but rather was cleaved to relatively large fragments. When cycloheximide was added to cells in S phase, the DNA synthesized during that cycle, both before and after the addition of the drug, was broken into pieces with a peak size at 4×10^6 daltons. The parental DNA in S-phase cells and nonreplicating DNA in G1 cells were also cleaved, but the pieces were longer with a broad peak centered at 7×10^6 daltons. The reason for these results is unclear, but the data suggest the presence of an in situ nuclease.

TABLE II: Temperature Effects on DNA Maturation.

Time at 27 °C (h)	Thymidine[a] Incorp (pmol/10^6 cells)	Mol Wt[b] ($\times 10^6$) 27 °C	Calcd Mol Wt[c] 37 °C
1	0.093	4	4
4	0.28	18	17
8	0.54	33	38

[a] The same cultures were used as in Figure 4. After incubating the cells at 27 or 37 °C for 45 min, 15 mL of cells (2×10^6/mL) was diluted with 85 mL of AA media containing 2.94 mg/mL [^{3}H]thymidine (40 μCi/mg) and maintained at 27 °C (○—○) or 37 °C (○—○). At each time point, 5-mL aliquots were assayed for acid-insoluble [^{3}H]thymidine. [b] From Figure 4. [c] Calculated by interpolation of data in Figure 4.

Effects of Reduced Temperature on DNA Replication. Since DNA replication consists of many steps, each theoretically with its own temperature dependence, it was postulated that decreasing the temperature might disrupt the balance between processes involved in DNA maturation and nucleotide polymerization and show a different character of the DNA growth as studied by the present procedure. To determine whether DNA chains synthesized at 27 and 37 °C were of equal length after equal amounts of total synthesis, cells were labeled with bromodeoxyuridine in early S phase and, after a media change, the culture was divided in half for incubations at these two temperatures. The growing points were marked with a pulse of [^{3}H]thymidine and replication continued with cold thymidine. After irradiation, the size of the new DNA was measured with alkaline sucrose gradients (Figure 4). Since the times selected for the size analysis did not correspond to equal amounts of total DNA synthesis, it was necessary to calculate the molecular weight of the DNA peak expected for the 37 °C samples after synthesizing the amount of DNA made in the 27 °C samples (Table II). When this was done it was found that, although the rate of DNA synthesis at 27 °C was approximately 20% of the initial rate of 37 °C, the chain lengths were very comparable for equivalent amounts of DNA synthesized. This result suggests that the limitation of DNA synthesis at lower temperatures is due to a lower rate of polymerization or small fragment initiation. This finding is in general agreement with the results of Huberman and Horwitz (1973) and Gautschi and Clarkson (1975). Since the DNA fragments in this experiment are approaching the size of replicons, the data apply only to the DNA replication process within a replicon; further investigation of more extended growth intervals may well unveil events in replicon termination or ligation that are uniquely temperature sensitive.

Discussion

In this study, we have combined the techniques of photolysis of bromodeoxyuridine-substituted DNA and alkaline sucrose gradient sedimentation analysis to explore the process of DNA replication. The ability to introduce controlled breaks in the DNA offers the following advantages: (1) DNA strand growth can be followed easily at any time during the S phase both in living cells and in subcellular preparations; (2) the complications associated with comparing small differences between a wide range of large DNA molecules are avoided by effectively having a zero molecular weight as a starting point; and (3) the smaller size of the fragments also makes them less prone to

potential hazards of the alkaline sucrose gradient analysis procedure such as incomplete denaturation by the alkali, entanglement of the strands, and speed dependence of the S value. In addition, the labeling procedure can be manipulated to offer a new probe for investigating the organization of the DNA replicating units in different regions of the genome. The fact that our data agree well with reports from other laboratories using different methods of analysis encourages the further use of the present approach in examining specific areas of DNA during replication in cells that have been submitted to certain inductive, differentiating, or viral stimuli. The present method of analysis already has proven useful in the study of DNA replication in subcellular systems where it has been used to show that DNA synthesis continues from sites which were initiated in vivo and that the nature of the chain growth is similar to that occurring in living cells (Planck and Mueller. 1977).

Two conclusions can be drawn from our investigation into the effect of cycloheximide on DNA replication: (a) the rate of strand elongation is decreased and (b) Okazaki or other small fragments do not accumulate. These observations indicate that inhibition of protein synthesis initially interferes with either initiation or elongation of Okazaki fragments. Further work is required to determine whether there are any unique effects of the initiation of specific replicons or the ligation of certain replicons during the maturation of the chromosomal DNA. Although the cycloheximide induces an immediate cessation in protein synthesis, there was a 10-min delay before DNA synthesis was inhibited. This can be explained by assuming that there is a limited pool of a specific protein that is used up by the process of DNA replication. This explanation is in accord with earlier data from this laboratory that indicated the presence of such a labile component in the nuclear DNA replicase system (Seki and Mueller, 1975). The present studies on chain growth would support the conclusion that such an entity is very critical to the overall functioning of the DNA replicase system since there was no imbalance of product formation noted in the chain growth analysis under conditions of restricted protein synthesis.

Acknowledgments

The authors are grateful to Drs. R. Wells, E. Calva, and M. Mulkins for their gifts of bacteriophage ϕX174 [^{3}H]DNA, T7 [^{3}H]DNA, and T4 containing [^{14}C]DNA. The authors thank Rebecca Aft for her technical assistance and M. LeMahieu, D. Schoenberg, and G. Planck for their assistance in the preparation of the manuscript.

References

Amaldi, F., Giacomoni, D., and Zito-Bignami, R. (1969), *Eur. J. Biochem. 11,* 419.

Cairns, J. (1966), *J. Mol. Biol. 15,* 372.

Callan, H. G. (1972), *Proc. R. Soc. London, Ser. B 181,* 19.

Friedman, D. L. (1974), *Biochim. Biophys. Acta 353,* 447.

Gautschi, J. R. (1974), *J. Mol. Biol. 84,* 223.

Gautschi, J. R., and Clarkson, J. M. (1975), *Eur. J. Biochem. 50,* 403.

Gautschi, J. R., and Kern, R. M. (1973), *Exp. Cell Res. 80,* 15.

Gautschi, J. R., Kern, R. M., and Painter, R. B. (1973), *J. Mol. Biol. 80,* 393.

Goldstein, N. O., and Rutman, R. J. (1973), *Nature (London), New Biol. 244,* 267.

Hand, R. (1975a), *J. Cell Biol. 64,* 89.

Hand, R. (1975b), *J. Cell Biol. 67,* 761.

Hand, R., and Tamm, I. (1972), *Virology 47,* 331.

Hand, R., and Tamm, I. (1973), *J. Cell Biol. 58,* 410.

Hershey, H. V., and Taylor, J. H. (1974), *Exp. Cell Res. 85,* 79.

Hori, T., and Lark, K. G. (1973), *J. Mol. Biol. 77,* 391.

Housman, D., and Huberman, J. A. (1975), *J. Mol. Biol. 94,* 173.

Huberman, J. A., and Horwitz, H. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 233.

Huberman, J. A., and Riggs, A. D. (1968), *J. Mol. Biol. 32,* 327.

Huberman, J. A., and Tsai, A. (1973), *J. Mol. Biol. 75,* 5.

Hyodo, M., Koyama, H., and Ono, T. (1971), *Exp. Cell Res. 69,* 461.

Kidwell, W., and Mueller, G. C. (1969), *Biochem. Biophys. Res. Commun. 36,* 756.

Lehmann, A. R. (1972a), *J. Mol. Biol. 66,* 319.

Lehmann, A. R. (1972b), *Biophys. J. 12,* 1316.

Lett, J. T., Klucis, E. S., and Sun, C. (1970), *Biophys. J. 10,* 277.

McFarlane, P. W., and Callan, H. G. (1973), *J. Cell Sci. 13,* 821.

McGrath, R. A., and Williams, R. W. (1966), *Nature (London) 212,* 534.

Mueller, G. C., and Kajiwara, K. (1966), *Biochim. Biophys. Acta 114,* 108.

Mueller, G. C., and Kajiwara, K. (1969), in Fundamental Techniques in Virology, Habel, K., and Salzman, N. P., Ed., New York, N.Y., Academic Press, p 21.

Mueller, G. C., Kajiwara, K., Stubblefield, E., and Rueckert, R. R. (1962), *Cancer Res. 22,* 1084.

Nuzzo, F., Brega, A., and Falaschi, A. (1970), *Proc. Natl. Acad. Sci. U.S.A. 65,* 1017.

Parkhurst, J. R., Peterson, A. R., and Heidelberger, C. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3200.

Planck, S. R., and Mueller, G. C. (1977), *Biochemistry 16* (in press).

Regan, J. D., Setlow, R. B., Kaback, M. M., Howell, R. R., Klein, E., and Burgess, G. (1971a), *Science 174,* 147.

Regan, J. D., Setlow, R. B., and Ley, R. D. (1971b), *Proc. Natl. Acad. Sci. U.S.A. 68,* 708.

Schandl, E. K., and Taylor, J. H. (1969), *Biochem. Biophys. Res. Commun. 34,* 291.

Seale, R. L., and Simpson, R. T. (1975), *J. Mol. Biol. 94,* 479.

Seki, S., and Mueller, G. C. (1975), *Biochim. Biophys. Acta 378,* 354.

Stubblefield, E., and Mueller, G. C. (1962), *Cancer Res. 22,* 1091.

Studier, F. W. (1965), *J. Mol. Biol. 11,* 373.

Taylor, J. H. (1959), *Proc. Int. Congr. Genet., 10th 4,* 63.

Weintraub, H. (1972), *Nature (London), New Biol. 236,* 195.

Weintraub, H., and Holtzer, H. (1972), *J. Mol. Biol. 66,* 13.

Werner, D., and Maier, G. (1975), *Eur. J. Biochem. 54,* 351.