DNA Chain Growth in Isolated HeLa Nuclei†

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ABSTRACT: A new method for studying DNA chain growth has been used to investigate DNA replication in isolated nuclei. For this purpose, DNA replication was initiated in S-phase cells with bromodeoxyuridine in order to photosensitize active replicons. The nuclei were then isolated and caused to synthesize DNA in vitro with [^{3}H]dTTP as a precursor to label growing chains. Subsequent irradiation with 313-nm light fragments the bromodeoxyuridine-DNA leaders and releases the contiguous, newly replicated DNA chains for analysis by sedimentation through alkaline sucrose gradients. This method of analysis provides data on the length of the DNA chain segments that are actually synthesized in vitro and on the fraction of these DNA chains that extends from bromodeoxyuridine-prelabeled replicons. Segments of 5 to 9 × 10^3 nucleotides attached to the bromodeoxyuridine leaders comprised approximately 40 to 50% of the DNA synthesized in the complete subcellular system. The remainder of the DNA consisted of short fragments of 6–13 S that were initiated in vitro, but failed to be ligated to longer strands. The latter appears to accumulate in the subcellular system as the result of a partial interruption of the native DNA replication process. Omission of the 105 000g cytoplasmic supernatant from the reaction mixture reduced the lengths of the DNA segments synthesized in vitro and the amount of DNA that was replicated. No significant amount of repair synthesis occurred on the template DNA and the initiation of replication in new replicons was restricted in the nuclear system. The data, however, support the concept that DNA synthesis in isolated nuclei continues largely from sites that were actively replicating in the living cells and that isolated nuclei provide a system for dissection of the molecular processes involved in the replication of chromosomal DNA.

Previous reports from this laboratory have described subcellular systems for studying the DNA replication process of eukaryotic cells (Friedman and Mueller, 1968; Kidwell and Mueller, 1969; Hershey et al., 1973; Seki et al., 1975; Seki and Mueller, 1975, 1976; Thompson and Mueller, 1975); when supplemented with optimum levels of the four deoxyribonucleoside triphosphates, Mg^{2+}, NaCl, ATP, and the soluble proteins of the cytoplasm, more than 2% of the chromosomal DNA could be replicated by nuclei isolated from S-phase HeLa cells. Studies with nuclei from cells in which replicating sites have been density labeled with bromodeoxyuridine have shown that a fraction of the DNA synthesized in vitro extends from the sites that were active in the living cells at the time of nuclear preparation. The present paper describes the nature of DNA chain growth in this system as revealed by a new procedure that photochemically releases the newly replicated DNA chains and permits their identification and size determination. This method of analysis has provided evidence that about one-half of the DNA synthesized in the complete system involves the elongation of the bromodeoxyuridine-labeled chains at native replication sites; the other half consists of DNA segments that are shorter than the average replicon and that fail to become ligated into mature, newly replicated DNA chains. The latter fragments may represent or derive from normal intermediates in the process of discontinuous replication of chromosomal DNA. Product analysis has also demonstrated that the soluble cytoplasmic proteins play a critical role in both chain elongation and the production of the short DNA segments. The initiation of new replicons (replicating units; Huberman and Riggs, 1968) in the nuclear system appears to be a limited process.

Materials and Methods

Cell Culture. Suspension cultures of HeLa cells were maintained in modified Eagle's medium, as described previously (Mueller et al., 1962). The cultures were enriched for cells in early S phase by treatment of logarithmically growing cells with amethopterin (1 μM) and adenosine (50 μM) to produce a thymidineless state. After 16 h, thymidine or bromodeoxyuridine was added, as indicated in the figure legends, to reverse the thymidineless state and permit the cells to resume DNA synthesis (Mueller and Kajiwara, 1969).

Cell Fractionation. Nuclei and the soluble cytoplasmic fractions were prepared according to the procedure of Hershey et al. (1973). In practice, 2 h after reversal of the thymidineless state, the cells were harvested and washed with cold hypotonic buffer A (10 mM Tris (pH 8, 22 °C), 4 mM $MgCl_2$, 1 mM EDTA, and 6 mM 2-mercaptoethanol) and resuspended in cold buffer A at 60 × 10^6 cells/mL. All of the following operations were carried out at 0–4 °C. The cells were lysed with a Dounce homogenizer (type B, Kontes Glass Co.) and centrifuged at 800g for 7 min to sediment the nuclei. The supernatant fraction was recentrifuged at 105 000g for 1 h to remove particulates and obtain a clear solution, which was used directly as the cytoplasmic fraction. The latter was generally used immediately, but occasionally was stored at −20 °C until needed. The nuclear pellet was washed three times with buffer A and resuspended in buffer A at 30 × 10^6 nuclei/mL for assay purposes.

DNA Replicase Reaction Conditions. The conditions for assaying replicase activity in nuclei were modified from those described by Hershey et al. (1973) so that each reaction mixture contained 50 μL of nuclear suspension (1.5 × 10^6 nuclei), 100 μL of cytoplasmic fraction or buffer A, and 75 μL of reaction mix containing 100 mM Tris (pH 8, 22 °C), 300 mM NaCl, 20 mM $MgCl_2$, 15 mM ATP, 0.3 mM dATP, 0.3 mM

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dCTP, 0.3 mM dGTP, 10–30 μM dTTP, and 250–450 μCi/mL [^{3}H]dTTP. The samples were incubated at 37 °C for 30 min unless otherwise indicated.

Analysis of DNA Size by Sedimentation. The molecular weight of single-stranded DNA was determined by sedimentation in alkaline sucrose gradients as described previously (Planck and Mueller, 1977). In practice, 37-mL gradients containing 0.3 M NaOH, 0.7 M NaCl, 1 mM EDTA, 60 mM sodium *p*-aminosalicylate, and 5–20% sucrose were prepared and allowed to sit overnight at 8 °C. One hundred microliters of the lysing solution (1 M NaOH–0.1 M EDTA) was layered on the gradients and followed immediately by 2–4 × 10^5 whole cells in 50 μL of SSC (0.15 M NaCl–15 mM sodium citrate) or 5–7 × 10^5 nuclei in 50 μL of the replicase assay mixture. The cells or nuclei were allowed to lyse for at least 30 min at 8 °C prior to centrifugation in a Beckman SW 27 rotor at 8 °C to a w^2t of 2.37 × 10^{11} rad^2/s. The actual speeds and times for specific experiments are shown 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 Soluene 100 (Packard). Toluene–PPO scintillation fluid was added, and the samples were left at room temperature for at least 8 h to decrease the chemiluminescence. 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, 16S; T7, 37S; T4, 70S. Molecular weights were calculated according to the relation derived by Studier (1965).

Photolysis of Bromodeoxyuridine-Labeled DNA Chains by Ultraviolet Irradiation. Fifty microliters of a replicase reaction mixture containing nuclei appropriately prelabeled with bromodeoxyuridine was irradiated with UV light as previously described (Planck and Mueller, 1977). For this purpose, the samples were placed in Linbro microtiter plate wells 5 cm below a Westinghouse Model FS20 sunlamp bulb. The light was filtered through a 1-cm deep layer of a thymidine solution (2 mg/mL) contained in the bottom half of 15 × 60 mm Falcon plastic tissue culture dish to remove the lower wavelength UV light and heat. Control experiments indicated that the standard irradiation treatment of 2 h at 4 °C was sufficient to fragment the BUdR-DNA in the isolated nuclei to pieces less than a few hundred nucleotides in length as reported previously for intact cells.

Results

Size of [^{3}H]DNA Labeled in Vitro and Its Covalent Linkage to DNA Labeled in Vivo with Bromodeoxyuridine. A method for studying the in situ elongation of DNA chains in living HeLa cells has been described elsewhere (Planck and Mueller, 1977). The method involves the labeling of active replicons with a pulse of bromodeoxyuridine followed by the growth of nascent DNA chains from a pool of [^{3}H]thymidine. Subsequent irradiation with 313-nm light fragments the BUdR-DNA[1] and releases the nascent TdR-DNA chains that extend from BUdR-DNA leaders. The released chains, in turn, can be sedimented as single-stranded DNA through an alkaline sucrose gradient for analysis of their size. In the present report, this method of analysis has been applied in a modified form to the characterization of DNA synthesized in isolated nuclei. The length of the DNA chains actually synthesized in vitro and the fraction of the DNA chains replicated in vitro that extend from bromodeoxyuridine-prelabeled replicons have been determined.

For these studies, cells which had accumulated in early S phase were released from a thymidineless state by adding the analogue, bromodeoxyuridine. The nuclei were isolated 2 h later when the cells were actively engaged in DNA replication. The nuclei were incubated in vitro in the presence or absence of the cytoplasmic fraction along with the appropriate substrates and ions to support the continuation of DNA replication. After 30 min, the overall length of the DNA chains was analyzed by sedimentation in alkaline sucrose gradients.

Approximately two-thirds of the DNA that was synthesized in the absence of the cytoplasmic fraction sedimented in alkaline sucrose gradients as short chains of 6–10 S (4–15 × 10^2 nucleotides); the remainder was distributed among segments that ranged from 20 to 80 S (Figure 1A). The presence of the cytoplasmic proteins in the reaction mixture increased the total amount of DNA synthesized sixfold and nearly doubled the fraction of isotope associated with higher molecular weight DNA (i.e., 20–80 S). The length of the DNA in the peak of short DNA chains near the top of the gradient was also increased to 10–13 S (1.5–3 × 10^3 nucleotides, Figure 1B); this shift, which was observed in repeated experiments, corresponds to a two- to fourfold increase in length and agrees with the threefold increase in the amount of associated radioactivity. From these data, it appears that one major function of the cytoplasmic proteins is to stabilize the active growing points which in turn results in the production of longer DNA chains. Another major function is to support the production of short DNA segments that are initially associated with the large template DNA, but are released in the alkaline gradients. This may involve a role in the initiation of these fragments.

When photolysis of the BUdR-DNA strands synthesized in the living cells was performed, the DNA segments formed by continued growth of these strands in the isolated nuclear system were released. This resulted in a shift of radioactivity in the sucrose gradients to correspond to shorter chain lengths. The resulting [^{3}H]DNA fragments sedimented to a peak at 6–10 S from nuclei incubated with buffer A alone (Figure 1C) and at 16–20 S from nuclei incubated with the cytoplasmic fraction (Figure 1D). The 6- to 12-fold increase in the length of the chains synthesized in the presence of the cytoplasmic fraction agrees again with the increase in total radioactivity that was found in these fragments.

The observation that very little radioactivity was associated with longer DNA segments after the UV irradiation rules out aberrant DNA repair synthesis or terminal addition on the bulk of the nuclear DNA as a significant factor in these experiments. The DNA synthesis in the nuclear system was further localized to sites that were actively replicating in vivo by an experiment in which cells in mid S phase were treated with

[1] Abbreviations used: BUdR-DNA, DNA containing 5′-bromodeoxyuridine; TdR-DNA, DNA containing deoxythymidine; Tris, Tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; SSC, 0.15 M NaCl–15 mM sodium citrate; PPO, 2,5-diphenyloxazole.

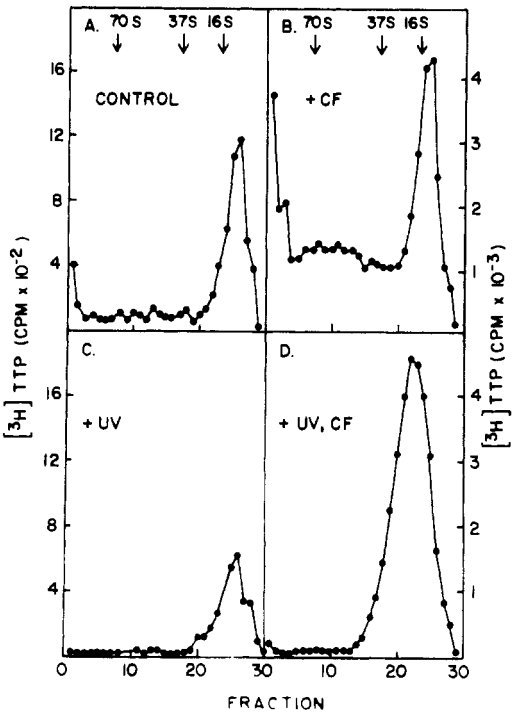

FIGURE 1: Alkaline sucrose gradient analysis of the size distribution of [^{3}H]DNA synthesized in isolated nuclei in the presence and absence of the cytoplasmic fraction. Nuclei were isolated from S-phase cells 2 h after DNA synthesis was initiated by the addition of bromodeoxyuridine (0.5 mg/L) to amethopterin blocked cells. To continue DNA replication in vitro, the isolated nuclei were incubated for 30 min at 37 °C in a DNA replicase reaction mixture containing 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 10 μM [^{3}H]dTTP (8.3 Ci/mmol), 5 mM ATP, 40 mM Tris (pH 8, 22 °C), 9.3 mM $MgCl_2$, 100 mM NaCl, 0.67 mM EDTA, and 4 mM 2-mercaptoethanol. The reaction mixtures were supplemented (B and D) or not supplemented (A and C) with the cytoplasmic fraction from cells reversed for 2 h with 0.5 mg/L thymidine. The size distribution of [^{3}H]DNA in 50-μL aliquots was analyzed on alkaline sucrose gradients before (A and B) and after (C and D) irradiation with UV light to fragment the BUdR-DNA leaders, as described under Materials and Methods. Centrifugation was for 15 h at 20 000 rpm in a Beckman SW27 rotor.

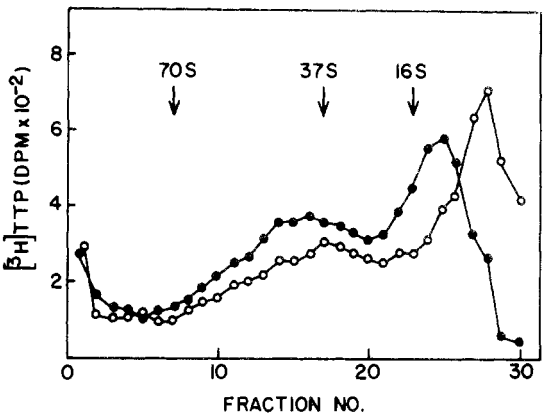

FIGURE 2: The analysis of the size distribution of DNA chains synthesized in nuclei pulse labeled with [^{3}H]dTTP and chased with nonradioactive dTTP. The nuclear and cytoplasmic fractions were isolated from S-phase cells 2 h after DNA synthesis was initiated by the addition of thymidine (0.5 mg/L) to amethopterin blocked cells. The nuclei were suspended in the DNA replicase reaction mixture (4 μM [^{3}H]TTP, 44 Ci/mmol) containing the cytoplasmic fraction and incubated for 45 s at 37 °C (O—O) to label the nascent DNA. A 250-fold excess of unlabeled dTTP was then added and the nuclei were incubated for an additional 15 min (●—●). The size distribution of [^{3}H]DNA in 50-μL aliquots of each sample was analyzed on alkaline sucrose gradients. Centrifugation was for 11.3 h at 23 000 rpm.

bromodeoxyuridine for only the 15 min immediately before the cells were harvested. With UV irradiation the radioactivity in the longer [^{3}H]DNA chains was also shifted to smaller sizes as observed in the previous experiment (data not shown). In addition, these results tend to exclude the activation of DNA synthesis in replicons that were for some reason dormant during the bromodeoxyuridine incorporation interval in living cells.

The rate of DNA chain growth in vitro can be determined by measuring the size of the [^{3}H]DNA released from bromodeoxyuridine-prelabeled DNA after pulse labeling of the active growing points with [^{3}H]dTTP and continuing replication with unlabeled dTTP for specified intervals. Preliminary experiments indicate that the DNA is growing at an overall rate of 7 to 10 × 10^4 daltons/min. This value is one-tenth to one-third the rate observed in living cells (Hori and Lark, 1973; Hand and Tamm, 1973; Housman and Huberman, 1975; McFarlane and Callan, 1973; Weintraub and Holtzer, 1972; Gautschi et al., 1973). It must be regarded as a minimal estimate for the rate of chain elongation in the nuclear system because any chain termination or interruption during the course of the experiment would substantially lower the observed growth rate due to separation of part of the product.

Mechanism of DNA Synthesis in Vitro. In living cells, DNA is synthesized on at least one parental strand as a series of short discontinuous pieces, Okazaki fragments, which subsequently are mature and ligated to yield the completed DNA strand (Schandl and Taylor, 1969; Nuzzo et al., 1970; Kidwell and Mueller, 1969; Goldstein and Rutman, 1973; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975; Okazaki et al., 1968). To determine whether DNA synthesis in vitro also proceeds with Okazaki fragment intermediates, the following type of experiment was done. DNA was synthesized with [^{3}H]dTTP for a 45-s interval in nuclei isolated from S-phase cells. As shown in Figure 2, the [^{3}H]DNA was distributed over a wide range of sizes, but a distinct peak was observed near the top of the gradient that corresponded to pulse-labeled Okazaki fragments of 2 to 6 S from living, S-phase HeLa cells (see Figure 3). With continued incubation in the presence of a 250-fold excess of nonradioactive dTTP to dilute the [^{3}H]dTTP pool, the shorter DNA chains labeled by the [^{3}H]dTTP pulse matured into DNA fragments of 6 to 8 S. Therefore, DNA segments that correspond to the size of Okazaki fragments in vivo are made in vitro and are also precursors for longer DNA chains of all size modes.

The persistence of the 10S peak (i.e., seen in the absence of UV irradiation) during the chase suggests that this DNA fragment may be an end product of the synthesis in the in vitro system. To assess more accurately whether or not the 10S DNA segments serve as precursors for the higher molecular weight DNA, nuclei from S-phase cells were allowed to replicate DNA with [^{3}H]dTTP for a 5-min interval to label the 10S DNA fragment (Figure 4). The pool of [^{3}H]dTTP was then diluted with a 100-fold excess of unlabeled dTTP, and the incubation was continued for an additional 25 min. During this interval, the distribution of the [^{3}H]DNA among the different chain lengths did not change appreciably. Thus, the 10S DNA segments were not ligated to form longer DNA chains either in the presence or absence of the cytoplasmic fraction. Furthermore, the size distribution of [^{3}H]DNA chains from nuclei labeled for the entire 30 min was also essentially identical with that of pulse-labeled nuclei. Accordingly, the 10S DNA must have been formed throughout the course of the incubation period at a rate that was tightly coupled to the growth of the

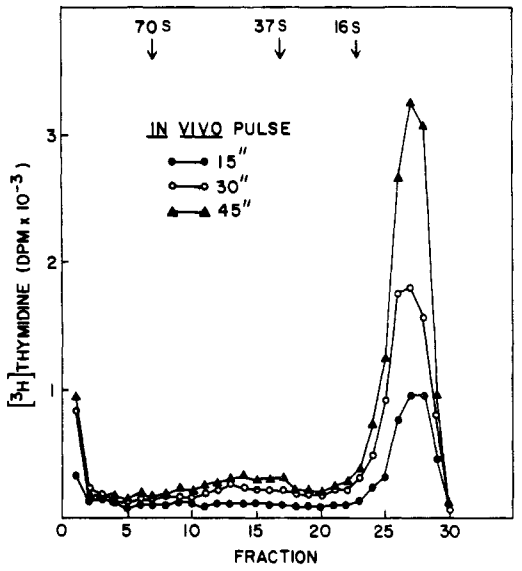

FIGURE 3: Demonstration of short DNA chains in cells pulse labeled with [^{3}H]thymidine in vivo. DNA synthesis was initiated in amethopterin-treated cells by adding bromodeoxyuridine (40 μg/10^8 cells). Two hours later the cells were harvested and resuspended at 2 × 10^6 cells/mL in AA media (fresh medium containing amethopterin (1 μM) plus adenosine (50 μM)). The cells were incubated for 30 min to exhaust RudR nucleotide pools and then exposed to 5 μCi/mL [^{3}H]thymidine (55 Ci/mmol) for 15 s (●—●), 30 s (○—○), and 45 s (▲—▲) to label the nascent DNA. The reaction was stopped by adding 20 volumes of cold SSC. The cells were harvested and resuspended in SSC (6 × 10^6/mL), and 50-μL aliquots were analyzed by lysis and sedimentation through alkaline sucrose gradients. Centrifugation was for 15.75 h at 19 500 rpm.

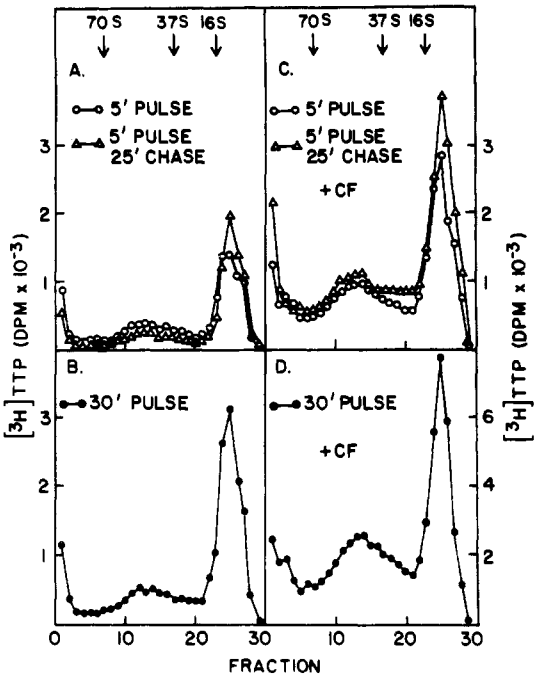

FIGURE 4: Analyses of DNA chain growth in nuclei pulse labeled with [^{3}H]dTTP. The nuclear and cytoplasmic fractions were isolated from cells which were 2 h into the S phase after the reversal of the aemethopterin block by the addition of thymidine (0.5 mg/L). In A and C, one set of nuclei (○—○) was pulse labeled for 5 min with [^{3}H]dTTP and the DNA analyzed directly by sedimentation in an alkaline sucrose gradient; a second set of pulse-labeled nuclei (△—△) received 1 mM dTTP (100-fold dilution) and was incubated for an additional 25 min prior to analysis of the DNA chain sizes. In B and D the nuclei were incubated for 30 min with [^{3}H]dTTP to label all of the newly replicated DNA. In all cases the size distribution of [^{3}H]DNA in 50-μL aliquots of each sample was analyzed on alkaline sucrose gradients. Centrifugation was for 11.3 h at 23 000 rpm.

longer chains that, as shown earlier, extend from sites that are active in vivo.

Discussion

A new method for analyzing DNA chain growth has been used to study DNA replication in isolated HeLa nuclei. The basic strategy involved labeling active replicons in S-phase cells with bromodeoxyuridine just prior to isolation of the nuclei and use of the latter to synthesize DNA in vitro with [^{3}H]dTTP. The size and the amount of newly replicated [^{3}H]TdR-DNA that was covalently linked to the BUdR-DNA leaders were determined from the shift of the [^{3}H]DNA to lower molecular weights when the BUdR-DNA was fragmented specifically by irradiation of the nuclei with 313-nm light.

The [^{3}H]DNA synthesized in vitro by this protocol can be divided into three size categories: one class of longer segments ranging from 20 to 80 S in size and two classes of short fragments corresponding to the two general size ranges reported for Okazaki or other small fragments in mammalian cells, 2 to 6 S and 6 S to 13 S (Schandl and Taylor, 1969; Nuzzo et al., 1970; Hyodo et al., 1971; Goldstein and Rutman, 1973; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975). The longer [^{3}H]DNA segments, which cover the size distribution for replicons in eucaryotic cells (Huberman and Riggs, 1968; Hori and Lark, 1973; Housman and Huberman, 1975; McFarlane and Callan, 1973; Hand, 1975), were sensitive to fragmentation by UV irradiation. This sensitivity proves that this fraction of the DNA was attached to DNA recently replicated in the living cells and supports the concept that DNA synthesis in vitro extends from sites that were active in living cells. The longest DNA chains which were synthesized in vitro approximate 1.5 × 10^7 daltons, which is equivalent to half the size of a small replicon (Huberman and Riggs, 1968; Hori and Lark, 1973; Housman and Huberman, 1975; McFarlane and Callan, 1973; Hand, 1975). This observation is in accord with the bidirectional mode of DNA replication in replicons centrally labeled with BUdR as shown for the living cells (Planck and Mueller, 1977). However, the limited size of these DNA segments suggests that DNA synthesis in isolated nuclei concerns primarily the completion of actively replicating replicons and that adjacent replicons, if initiated and replicated in vitro, very likely do not become ligated to these segments. Currently, a number of modifications in the system that prolong the active life of replicase complexes are being investigated in this laboratory in the attempt to find conditions for the initiation and successive synthesis of complete replicons in vitro.

The 2-6S DNA, corresponding to the classical Okazaki fragments seen in pluse-labeling experiments in vivo, was observed only with very short labeling intervals in isolated nuclei. It is of considerable interest, however, that these fragments appeared to mature into both 20-80S and 6-13S DNA classes. Three possible explanations for this result are suggested by the bidirectional nature of the DNA replication process. In one situation the 2-6S nucleosome-size fragments may be produced at every growing point but that the ligation of these fragments proceeds differently along one of the opposing template strands to give rise to the 6-13S pieces. The observation that the amount of newly synthesized 6-13S DNA is nearly equivalent to the amount of 20-80S labeled DNA is in agreement with this concept. This assumes that maturation of Okazaki fragments in the overall 3′ to 5′ direction involves at least a one step difference from the process in the 5′ to 3′ direction. Alternatively, DNA replication might proceed by

a continuous mechanism on the other side (Friedman, 1974; Hershey and Taylor, 1974; Krokan et al., 1975; Kuebbing et al., 1976; Tseng and Goulian, 1975). In this case some impairment at the replication forks when the chains reach a certain size could give rise to the production of the 6–13S pieces. The third possibility is that an alkali-labile unit links the immature 6–13S DNA chains along one arm of the replication fork. Current experimentation is directed to a search for a limiting component in the growth of these fragments into replicon size chains and the characterization of the 5′ ends of these fragments.

The major role of the cytoplasmic proteins in the nuclear DNA replication system appears to involve the stabilization and maintenance of active growing points, particularly for the longer DNA chains. These proteins may be involved in the preservation and formation of new chromatin. In accord with this view, the fractionation of the cytoplasmic proteins has revealed that the activity resides in a series of distinct components which work cooperatively to support DNA replication in isolated nuclei; these same components bind efficiently and nonrandomly to chromatin (Kajiwara et al., 1976). The possibility that these proteins may change the frequency of initiation and termination of DNA synthesized in the nuclear system through interaction with specific chromosomal sites is being explored.

Acknowledgments

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