Kinetics of DNA Replication in C3H 10T1/2 Cells Synchronized by Aphidicolin†

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ABSTRACT: Aphidicolin is an inhibitor of DNA polymerase α and blocks nuclear DNA replication without interfering with mitochondrial DNA synthesis. The efficacy of this mycotoxin as a tool in cell synchronization was evaluated in C3H 10T1/2 clone 8 cells. At concentrations of 1–2 μg/mL, aphidicolin quickly reduced the [^{3}H]thymidine uptake to less than 5% of control levels in the first 5 min of incubation. This inhibition was easily reversed by washing and refeeding cells with fresh medium. The synchronization protocol consisted of first blocking cells by confluence arrest, replating them at lower density, and then treating the cells with aphidicolin for 24 h. Once the inhibitor was removed, DNA replication started without any delay. The cell population traversed the S phase in about 8 h and synchronously doubled in cell number. Autoradiography studies revealed a labeling index of 89–93% during the S phase. However, it was also observed that 10T1/2 cells were able to enter S phase in the presence of aphidicolin. The extent of the ensuing replication in the nucleus was dependent on the time that cells remained arrested in early S phase. Analyses of the newly replicated DNA in alkaline sucrose gradients revealed a fairly homogeneous distribution of sizes of nascent DNA in synchronized cells pulse-labeled at the beginning of the S phase. Upon chase in nonradioactive medium, the average molecular weight of the nascent DNA increased linearly with time of DNA synthesis for 2 h. The apparent rate of DNA chain growth determined from pulse and chase experiments was 1.2 μm/min. This rate was strongly inhibited (93%) by aphidicolin at a concentration of 2 μg/mL.

The replication of nuclear DNA in a eukaryotic cell is limited to the S phase. This period of the cell cycle can last from few minutes to several hours depending on the organism and its developmental stage, the degree of cellular differentiation, and, in culture, the growing conditions. The published values for observed rates of displacement of replication forks (0.1–2.5 μm/min) alone cannot explain these differences in the overall rate of DNA synthesis. However, it is well-known that numerous and independent units of DNA replication can be active concurrently within the eukaryotic nucleus during the S phase. The number of these units of replication and their average size and kinetics of activation are believed to be the critical factors in the regulation and organization of DNA replication [see reviews by Edenberg & Huberman (1975), Baserga (1976), Hand (1978), Sheinin et al. (1978), DePamphilis & Wassarman (1980), Harland (1981), and Kapp & Painter (1982)].

The kinetics of synthesis of nascent DNA within each of the replication units and the rate of size maturation of the newly replicated DNA have been studied in several organisms (Kowalski & Cheevers, 1976; Painter & Young, 1976; Lanotte et al., 1977; Johnston & Williamson, 1978; Funderud et al., 1978). With cells in culture, these studies would be facilitated by synchronization methods that could arrest cells at the G_1/S boundary and, thus, maximize the degree of synchrony of the population through the S phase. High concentrations of thymidine, hydroxyurea, and methotrexate have been used in attempts to block cells at the G_1/S boundary. These compounds have already been shown to allow initiation of S-phase DNA synthesis [see review by Hochhauser et al. (1981)]. Also, they inhibit DNA replication by depleting the cells of precursor molecules. This mechanism of action constitutes an obvious problem to studies that later require quick reversal of the inhibition of DNA synthesis and incorporation of label into replicating DNA. The fact that aphidicolin specifically and reversibly inhibits DNA polymerase α (Ikegami et al., 1978), without interfering with RNA and protein synthesis or with the expansion of nucleotide pools as cells prepare to enter S phase, favors its use in synchronization (Pedrali-Noy et al., 1980).

In this paper, we show that the inhibition of DNA polymerase α by aphidicolin also fails in blocking cells at the G_1/S boundary but arrests cells in early S phase. It does poise clusters of replicons ready to synthesize DNA once the inhibitor is removed. Therefore, it facilitates studies of elongation of the first group of replicons activated in early S and the maturation to high molecular weights. Furthermore, the data also allow estimations of DNA chain growth in 10T1/2 cells under these culture conditions.

Experimental Procedures

Cell Culture. C3H 10T1/2 clone 8 cells (Reznikoff et al., 1973) were a gift from Dr. Craig Boreiko (Chemical Industry Institute of Technology, Research Triangle Park, NC). They were grown as previously described (Greenberg et al., 1978) except that gentamicin was omitted in the stock cultures. No mycoplasma contamination has been detected in periodic tests of these cultures (Chen, 1977).

DNA Synthesis Inhibition. Aphidicolin was obtained from the Natural Products Branch, Division of Cancer Treatment, N.C.I., and dissolved at 2.5–5.0 mg/mL in dimethyl sulfoxide (Me_2SO). Logarithmically growing cells were treated with 2 μg/mL aphidicolin or the corresponding concentration of Me_2SO (0.08%) for 5–60 min. During the last 5 min of the desired incubation period, [^{3}H]thymidine was added to a final

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concentration of 10 μCi/mL. At the end of this pulse, each plate was washed 2 times with ice-cold Hanks' balanced salt solution (HBSS) and treated with 5% trichloroacetic acid (Cl_3CCOOH) at 4 °C for at least 30 min. Plates were then washed 3 times with ice-cold 5% Cl_3CCOOH and 3 times with 95% ethanol and air-dried. Cells were lysed in 1.5 mL of 0.3 N NaOH and incubated overnight at 37 °C. The absorbance at 260 nm was measured in each cell lysate and radioactivity determined in 1-mL aliquots after mixing with 120 μL of 3 N HCl. The cpm/A_{260nm} ratio was considered to reflect the amount of [^{3}H]thymidine incorporated into acid-insoluble products (Cordeiro-Stone et al., 1979) normalized for the number of cells in each plate (directly proportional to the absorbance of the lysate). For each time point, the average of triplicate plates was compared with the corresponding value for control plates, and the residual DNA synthesis was expressed as the percent of control. The kinetics of recovery of DNA synthesis from aphidicolin inhibition was followed after cells were treated for 30 min with the inhibitor. Cells were washed twice, refed with fresh medium, and pulse-labeled for 15 min with 2 μCi/mL [^{3}H]thymidine at different times following the removal of aphidicolin. Plates were processed as described above, and the DNA synthesis activity (cpm/A_{260nm}) was expressed as the percent of control.

Cell Synchronization. Cells were plated at 5000 cells/cm^2 in 100-mm plates and refed 3 days later. One week after being plated, cells were confluent, and their growth was arrested. They were then replated in 60-mm plates at 13 100 cells/cm^2. Aphidicolin was added to the culture medium (1–2 μg/mL, 0.08% Me_2SO) at the time of replating or 1–7 h later. After a 24-h incubation, the cells were washed and refed with fresh medium without aphidicolin. DNA synthetic activity was followed by incubating cells with 2 μCi/mL [^{3}H]thymidine for 30 min starting at different times following release from the aphidicolin block. Triplicate plates were used for each time point and processed as described above for determination of the cpm/A_{260nm} ratio. Identically treated plates were used to follow the growth of 10T1/2 cells submitted to the above synchronization procedure. Following trypsinization, the number of cells per 60-mm plate was determined in a Coulter counter.

Autoradiography. 10T1/2 cells were plated into Lab-Tek tissue culture slides (one or two chambers per slide) and treated with aphidicolin as described above. At the end of the [^{3}H]thymidine pulse (15 or 30 min), cells were fixed with methanol/acetic acid (3/1) as described by Stein & Yanishevsky (1979). Their procedures were also followed for dipping the slides in Kodak NTB-2 photographic emulsion, developing the autoradiograms, and poststaining with Giemsa. Two to four identical slides were prepared for each time point. The percent of labeled nuclei was scored with a light microscope at 450×.

Radioactive Labeling of DNA. Cells were synchronized as described above and treated to analyze the DNA synthesized in the presence or absence of aphidicolin. For analysis of aphidicolin-resistant synthesis, cells were exposed to 25–50 μCi/mL [^{3}H]thymidine during 21 of the 24-h incubation with 2 μg/mL aphidicolin (see Results). For analysis of postsynchronization DNA synthesis, cells were incubated for 3–120 min with 20–50 μCi/mL [^{3}H]thymidine immediately after removal from the aphidicolin-containing medium. When chase in nonradioactive medium followed a 3–15-min pulse with [^{3}H]thymidine, the cells were incubated in fresh medium containing thymidine and deoxycytidine at 1×10^{-5} M each. In some of the experiments, cells treated as above had been previously incubated with 0.005 μCi/mL [^{14}C]thymidine for at least 48 h during the first 3 days of logarithmic growth preceding confluence arrest.

Alkaline Sucrose Gradients and Molecular Weight Determinations. Cells in 60-mm plates were washed twice with ice-cold saline and scraped into 0.1 M NaCl and 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 8.0. Cells were passed 5 times through a 22- or 25-gauge needle to break up clumps. The cell suspension containing approximately 1.8×10^5 cells in 0.5 mL was then added to the lysis layer (0.5 mL of 1 M NaOH and 0.02 M EDTA) on top of a 36-mL linear sucrose gradient (5–20%) containing 0.4 M NaOH, 2.0 M NaCl, and 0.01 M EDTA. The gradients were left under standard fluorescent illumination in the laboratory for 1 h to facilitate cell lysis and DNA unwinding (Lehmann, 1981). The gradients were centrifuged in an SW27 rotor at 25 000 rpm and 20 °C for 4 h. Equal-volume fractions were collected from the bottom of the tube. Acid-insoluble radioactivity was determined in each fraction in the presence of carrier DNA by retention of the precipitate in Whatman GF/A filters. Radioactivity was measured by liquid scintillation; in double-labeling experiments, the ^{3}H counts were corrected for the spillover of ^{14}C radiation. The recovery of DNA from the gradients averaged 96 ± 11%. These gradients were calibrated with supercoiled and linear SV40 DNA, and the average molecular weight (M_a) of the DNA distributions was determined by the equation $M_a = \sum r_i M_i / \sum r_i$ where r_i is the radioactivity in fraction i and M_i is the mean molecular weight of DNA in fraction i as calculated from the sedimentation coefficient (S_i) by the equation $S_i = 0.0528 M_i^{0.400}$ (Studier, 1965). Given the conditions described for our experiments, M_a should correspond to the weight-average molecular weight (M_w) of uniformly labeled nuclear DNA, of DNA labeled in the presence of aphidicolin, or of nascent DNA labeled for 3–120 min after release from aphidicolin. This is because in these cases the DNA molecules are labeled their entire length. In pulse and chase experiments, however, with time, r_i becomes proportional to the number of labeled molecules in fraction i, rather than the weight of the DNA. Therefore, M_a as calculated should approximate the number-average molecular weight (M_n). Considering these difficulties, inherent to any molecular weight determination, in this paper, we will refer to the average molecular weight as M_a, determined as described above. Under the conditions described here, the uniformly labeled, parental DNA sediments in the alkaline sucrose gradients with an M_a of 2.96 (±0.15) × 10^8.

Results

Inhibition of Nuclear DNA Synthesis by Aphidicolin. As already shown by many authors [see review by Spadari et al. (1982)], aphidicolin is a potent inhibitor of nuclear DNA replication in eukaryotic cells but does not interfere with mitochondrial DNA synthesis (Zimmerman et al., 1980; Geuskens et al., 1981). Accordingly, the ability of C3H 10T1/2 cells to incorporate [^{3}H]thymidine into acid-insoluble products was drastically reduced in the presence of aphidicolin but never completely abolished. A residual synthesis representing 1–2% of control levels was observed even in the presence of 2–10 μg/mL aphidicolin. Also, the kinetics of inhibition was extremely fast, and DNA synthesis dropped to its minimal level in the first 5 min of incubation containing this mycotoxin (Figure 1). Nonetheless, the inhibition of replication by aphidicolin could be promptly reversed by washing the cells and refeeding them with fresh medium. The rate of recovery, however, was dependent on the concentration of aphidicolin initially used. After treatment with 2 μg/mL,

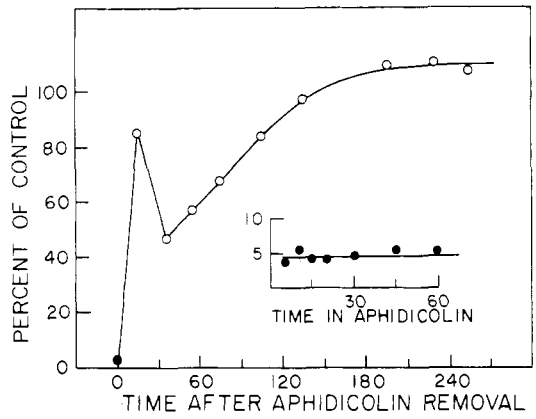

FIGURE 1: Kinetics of inhibition and recovery of DNA synthesis after treatment with aphidicolin. Logarithmically growing C3H 10T1/2 cells were treated with 2 μg/mL aphidicolin and pulse-labeled during the last 5 min of the indicated times in the presence of the inhibitor (●). Cells treated with aphidicolin for 30 min were washed twice, refed with fresh medium, and pulse-labeled during the last 15 min of the indicated times after aphidicolin removal (○).

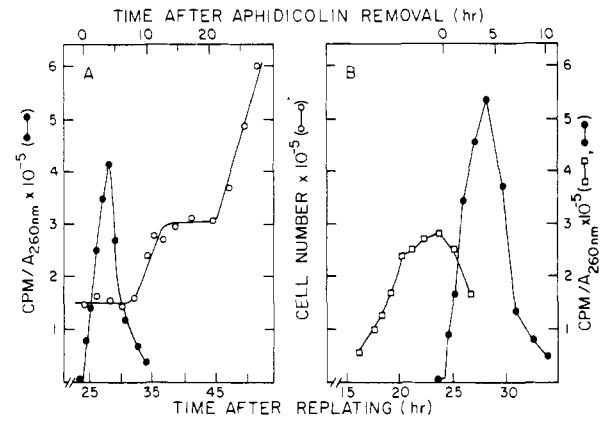

FIGURE 2: Synchronization of C3H 10T1/2 cells by aphidicolin. Cells were released from postconfluence inhibition of cell division and replated in medium containing 1 (A) or 2 μg/mL (B) aphidicolin. After 24 h, cells were washed and refed with fresh medium without aphidicolin. DNA synthetic activity was then followed by 30-min pulses with 2 μCi/mL (59 Ci/mmol) [^{3}H]thymidine at the indicated times following removal of aphidicolin (●). The results are expressed as the ratio of total acid-insoluble radioactivity to the absorbance at 260 nm of the cell lysate in 0.3 N NaOH. Each point represents the average of three plates. The point before time zero corresponds to [^{3}H]thymidine incorporation by the same cells, in the presence of aphidicolin, 30 min before they were released from the aphidicolin block. In (A), cell division was also followed after synchronization with 1 μg/mL aphidicolin (○). The DNA synthetic activity of control cells released from confluence inhibition but not treated with aphidicolin is also depicted in (B) (□).

the treated cells returned to control levels of [^{3}H]thymidine incorporation in about 2 h (Figure 1). Autoradiographic studies revealed that this rate of recovery was not the consequence of an increase in the percentage of cells synthesizing DNA but rather was due to the ability of each cell to incorporate [^{3}H]thymidine (supposedly reflecting the rate of dilution of aphidicolin inside each cell). After treatment with 4–10 μg/mL aphidicolin, the overall rate of DNA synthesis was not completely recovered, even after several hours of incubation in fresh medium (not shown). On the basis of these results, we selected 1–2 μg/mL aphidicolin as the optimal concentrations to inhibit nuclear DNA synthesis in 10T1/2 cells. Under these conditions, the incorporation of [^{3}H]thymidine into cellular DNA was reduced to 2–4% of the control.

Synchronization of C3H 10T1/2 Cells by Aphidicolin. Cells were arrested by postconfluence inhibition of cell division and then released by replating in medium containing aphidicolin at 1 μg/mL (Figure 2A) or 2 μg/mL (Figure 2B). After a 24-h incubation, the cells were washed free of the inhibitor and refed with fresh medium. The first wave of DNA synthesis and the number of cells per plate were followed at different times after release from the aphidicolin block (Figure 2A). The DNA synthetic activity of control cells released from confluence inhibition but not treated with aphidicolin is also shown in Figure 2B. These results do not represent the entire wave of DNA synthesis but confirm those published previously by Grisham et al. (1980). Basically, cells released from confluence arrest began to incorporate [^{3}H]thymidine at 15–17 h after replating. The subsequent wave of DNA synthesis extended over 17 h as a consequence of the low rate of entry of cells in S phase (Grisham et al., 1980). In the presence of aphidicolin, cells were able to progress through G_1, and they accumulated at the beginning of the S phase. As soon as aphidicolin was removed, the rate of incorporation of [^{3}H]-thymidine increased very quickly, reached a peak between 3 and 4 h later, and by 8 h was down to values lower than those observed between 0 and 30 min. The cell growth curve (Figure 2A) shows that the cell population remained constant in number during the entire period of DNA synthesis and started to divide after 8 h following release from the aphidicolin block. It took about 5 h for the cell population to completely double in number. This was followed by another plateau phase of about 7 h after which the cell population started another wave of cell division and again doubled in number. By taking the midpoints in the two doubling phases of the growth curve, we could roughly estimate a 15-h population doubling time, a value close to that observed for logarithmically growing populations of 10T1/2 cells (Reznikoff et al., 1973).

The synchronization protocol described above was also applied to cells plated directly on microscopic slides. The percentage of S-phase cell was then determined by autoradiography. The results in Figure 3A show that almost 70% of the cells were able to synthesize DNA immediately after removal of the aphidicolin block. It is noteworthy that control slides not exposed to aphidicolin revealed an identical number of cells in S at the same chronological time as the aphidicolin-treated cells. This means that every cell ready to synthesize DNA was able to do so as soon as aphidicolin was removed from the medium. In this particular experiment, the control culture never displayed a labeling index higher than 77%, while the one treated with aphidicolin reached a maximum of 89% of labeled nuclei 4 h after removal of the inhibitor. Therefore, we attempted to improve the degree of synchronization in early S phase by extending the aphidicolin treatment to allow all cycling cells to reach S phase before removing the block. Thus, aphidicolin was added for 24 h between 7 and 31 h after cells were replated from confluence-arrested cultures (Figure 3B). Under these conditions, the labeling index was 91 (±2%) from the time of aphidicolin removal until 4 h later. It then started to drop very quickly, reaching 11% by 8 h. This seemingly shorter S phase prompted us to analyze the rate of entry of cells in S phase in the presence and absence of aphidicolin, following release from growth arrest. Cells were replated in Lab-Tek slides, and 7.5 h later, 0.25 μCi/mL [^{3}H]thymidine (control) or 2 μg/mL aphidicolin together with 25 μCi/mL [^{3}H]thymidine was added to the culture medium. Afterward, cells were fixed at different times and labeled nuclei scored by autoradiography. The results shown in Figure 3C demonstrate that 10T1/2 cells were able to enter the S phase with approximately the same rate in the presence or absence of aphidicolin. These results are similar to those recently described by D'Anna & Tobey (1984) for Chinese hamster (line

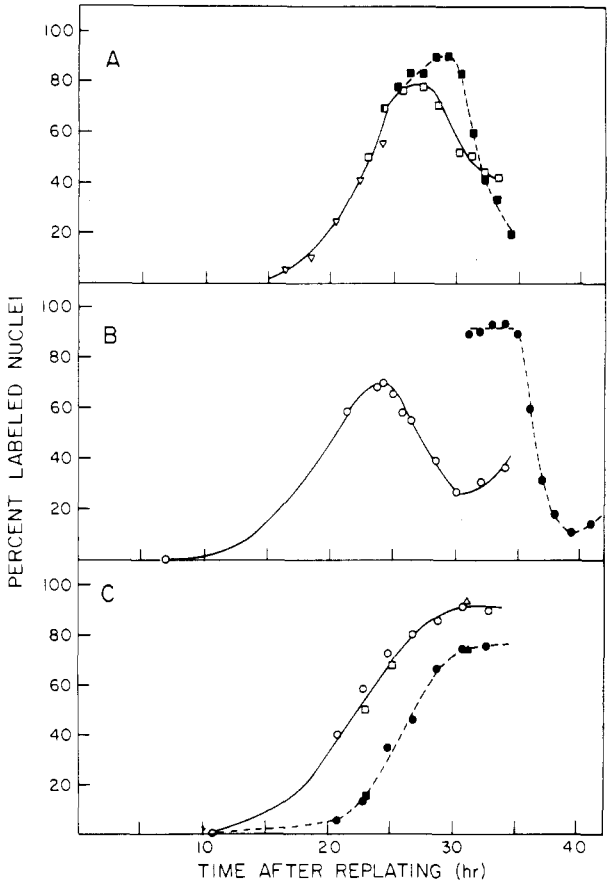

FIGURE 3: Determination of the frequency of cells in S phase by autoradiography. Confluence-arrested cells were replated directly on chambered slides and treated with 2 μg/mL aphidicolin for 1–24 (A), 7–31 (B), or 7–34 h (C) after replating (except for the control cells). Cells were pulsed with [^{3}H]thymidine for 30 (A) or 15 min (B) at different times after replating (control, open symbols) or after releasing from the aphidicolin blck (closed symbols). In (C), cells were continuously labeled with 0.25 μCi/mL [^{3}H]thymidine in the presence of 0.08% Me_2SO (control, open symbols) or 25 μCi/mL [^{3}H]thymidine in the presence of aphidicolin (closed symbols). Different symbols denote independent experiments.

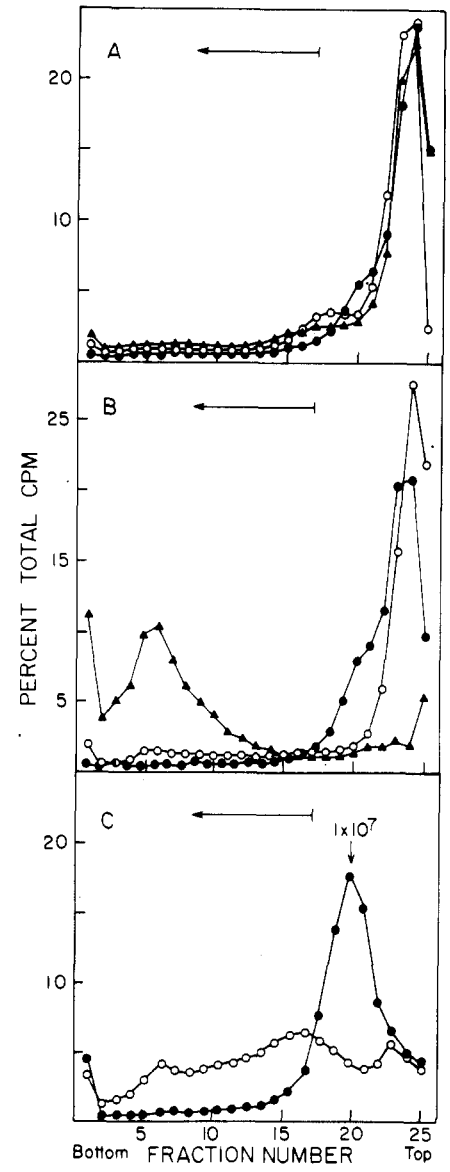

FIGURE 4: Characterization of aphidicolin-resistant DNA synthesis in alkaline sucrose gradients. Confluent cells were replated and treated with 2 μg/mL aphidicolin for 24 h at 1–25 (A), 4–28 (B), or 7–31 h (C) after replating. [^{3}H]Thymidine was added 1 h after the beginning of the aphidicolin treatment and removed 2 h before its end. Cells were released from the aphidicolin block and immediately lysed on top of alkaline gradients or first incubated in fresh medium for different periods of time. (A) Cells were labeled with 50 μCi/mL (15 Ci/mmol) [^{3}H]thymidine and then washed and chased for 0 [(●) total cpm = 19 381], 30 [(○) total cpm = 19 497], or 60 min [(▲) total cpm = 16 485]. (B) Cells were labeled with 25 μCi/mL (71 Ci/mmol) [^{3}H]thymidine and chased for 0 [(●) total cpm = 36 485] and 180 min [(○) total cpm = 35 115]. The percent distribution of uniformly labeled [^{14}C]DNA from the same cells chased for 180 min is also shown [(▲) total cpm = 1937]. (C) Cells labeled as in (B) and chased for 0 [(●) total cpm = 332 323; the molecular weight determined for the peak fraction is indicated] and 120 min [(○) total cpm = 350 586]. Alkaline sucrose gradients were prepared and run as described under Experimental Procedures. Sedimentation is from right to left as indicated by the arrows.

CHO) cells. We interpreted the displacement to the right of the curve representing aphidicolin-treated cells as due to the strong inhibition of DNA chain growth by aphidicolin (see below) and the difficulty in discriminating very low DNA synthetic activity in the nucleus against the background of cytoplasmic labeling. Since the mitochondrial DNA polymerase is not inhibited by aphidicolin, 100% of the cells were labeled in the cytoplasm. Only when the density of silver grains over the nucleus surpassed the one seen over the cytoplasm could that cell be unequivocally scored as having entered the S phase.

Characterization of DNA Synthesis Resistant to Aphidicolin. Cells released from confluence arrest were treated with 2 μg/mL aphidicolin for 24 h but beginning 1, 4, or 7 h after replating. One hour after the beginning of the aphidicolin treatment, [^{3}H]thymidine was added to the medium for 21 h. Then cells were washed free of the radioactive precursor but kept in aphidicolin for the remaining 2 h in order to chase any tritiated DNA precursor molecules from the soluble pools. When released from the aphidicolin block, cells were incubated in fresh medium (chased) for different periods of time before they were harvested and lysed on top of alkaline sucrose gradients. Figure 4 shows that prior to chase the majority of the [^{3}H]thymidine incorporated in the presence of aphidicolin was associated with small DNA molecules. However, the size distribution, as well as the proportion of these small molecules that could be chased to higher molecular weight DNA, was dependent on the timing of the aphidicolin treatment. In the experiment shown in Figure 4A, cells were incubated with aphidicolin at 1–25 h and [^{3}H]thymidine 2–23 h after replating. It is seen that, although the majority of the radioactivity stayed at the top of the gradient, a small shoulder, centered on fraction 20, was displaced to higher molecular

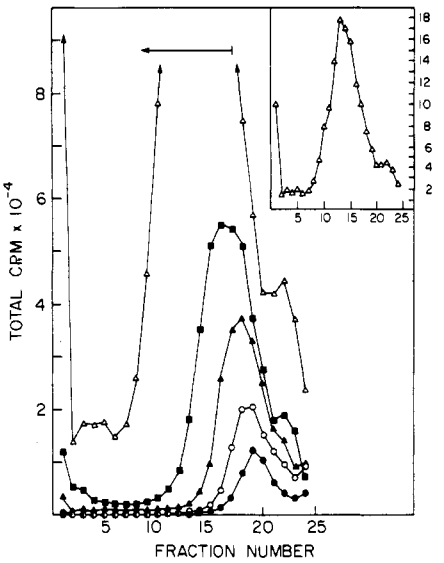

FIGURE 5: Distribution in alkaline sucrose gradients of labeled nascent DNA from synchronized cells. Cells were synchronized by treatment with 2 μg/mL aphidicolin as described in the legend of Figure 2B. Immediately after being released from the aphidicolin block, cells were incubated in medium containing 20 μCi/mL (71 Ci/mmol) [^{3}H]-thymidine for 10 [(●) total cpm = 56672], 20 [(○) total cpm = 120590], 30 [(▲) total cpm = 236577], 60 [(■) total cpm = 447213], or 120 [(△) total cpm = 1692976]. Total acid-insoluble radioactivity per fraction was plotted in this figure instead of the percent of total cpm. The inset shows the entire profile of nascent DNA labeled for 120 min.

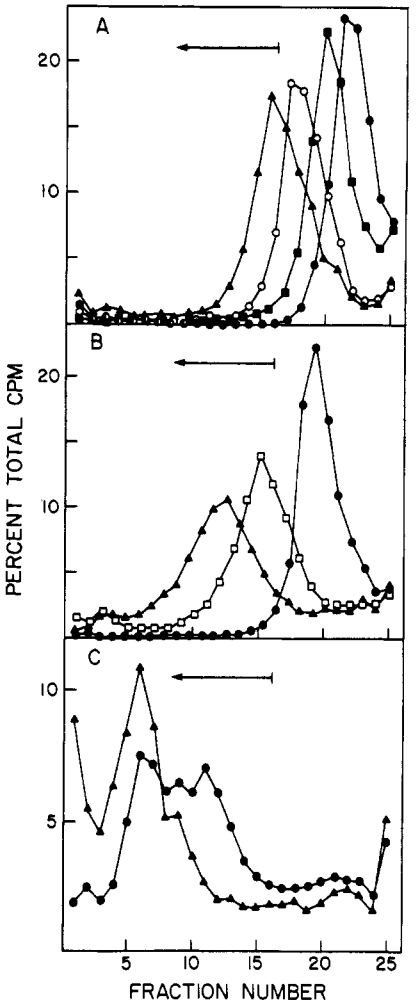

FIGURE 6: Percent distribution in alkaline sucrose gradients of nascent DNA labeled at the beginning of the S phase. Cells were synchronized by treatment with 2 μg/mL aphidicolin as described in the legend of Figure 2B and pulse-labeled immediately after being released from the aphidicolin block. (A) Cells were pulse-labeled for 3 min [(●) total cpm = 1434] with 50 μCi/mL (15 Ci/mmol) [^{3}H]thymidine and then chased for 27 [(○) total cpm = 4024] or 57 min [(▲) total cpm = 3624]. The distribution of labeled nascent DNA after a 10-min pulse with 20 μCi/mL (71 Ci/mmol) [^{3}H]thymidine is also shown [(■) total cpm = 56672]. (B) Cells were only pulse-labeled for 15 min with 25 μCi/mL (71 Ci/mmol) [^{3}H]thymidine [(●) total cpm = 81874] or pulse-labeled for 10 min with 20 μCi/mL (71 Ci/mmol) [^{3}H]thymidine and chased for an additional 65 [(□) total cpm = 27560] or 110 min [(▲) total cpm = 11877]. (C) Cells were pulse-labeled for 15 min with 25 μCi/mL (71 Ci/mmol) [^{3}H]thymidine and then chased for additional 165 min [(●) total cpm = 67968]. The percent distribution of the parental DNA, uniformly labeled with [^{14}C]thymidine in these same cells, is also shown [(▲) total cpm = 1994].

weights upon chase for 30–60 min. This shoulder was even more evident when cells were labeled from 5 to 26 h (Figure 4B) and dominated the distribution profile when cells were labeled from 8 to 29 h after replating (Figure 4C). Likewise, the proportion of labeled DNA chased to higher molecular weights were more prominent in Figure 4C than in Figure 4A. These results indicate an increased synthesis of nuclear DNA with time of incubation in aphidicolin and are in agreement with those in Figure 3C. We have also determined by a Hirt extraction procedure (Hirt, 1967) that 80–85% of the radioactivity incorporated during the 21-h incubation with [^{3}H]-thymidine, in a protocol identical with that in Figure 4B, was not associated with nuclear DNA (results not shown). It has been documented that the Hirt extraction procedure, based on the preferential precipitation of undegraded nuclear DNA in the presence of SDS and NaCl, allows the separation of mitochondrial DNA from nuclear chromatin (Radsak & Schutz, 1978). Our results are consistent with the conclusion that initially most of the DNA synthesis occurring in the presence of aphidicolin is due to the uninhibited replication of mitochondrial DNA in the cytoplasm (Geuskens et al., 1981). However, as the number of cells reaching the S phase increases in the population (Figure 3C), the nuclear DNA synthesized in the presence of aphidicolin predominates over the mitochondrial DNA (Figure 4C). Although aphidicolin inhibits DNA elongation very efficiently (Figure 1, also see below), it apparently has no effect on the initiation of the first replicons activated very early in S phase. An accumulation of replicon-sized intermediates in the presence of aphidicolin has also been described in asynchronous cultures of rat fibroblasts (Yagura et al., 1982).

Synthesis of Nascent DNA in 10T1/2 Cells Synchronized by Aphidicolin. Figure 5 shows the results of the distribution in alkaline sucrose gradients of nascent DNA molecules labeled continuously for 10, 20, 30, 60, or 120 min after cells were released from the aphidicolin block following the same protocol as in Figure 2B. The total cpm per fraction was plotted in Figure 5 in order to display the absolute increase in the amount of labeled nascent DNA of different molecular weights. The average molecular weight (M_a) of the population of nascent molecules increased steadily with time of DNA synthesis with no clear pause in chain growth. The M_a values for the profiles shown in Figure 5 were 1.9×10^7 (10 min), 2.4×10^7 (20 min), 3.3×10^7 (30 min), 7.1×10^7 (60 min), and 1.1×10^8 (120 min). The results in this experiment also reflect the continuous initiation of replicons during the time of incubation with [^{3}H]thymidine. These initiations are probably due to the activation of new replicon clusters and to a smaller degree to cells entering S phase at different times (see Figure 3A). To better follow the growth of the nascent DNA molecules labeled right at the beginning of the S phase, pulse and chase ex-

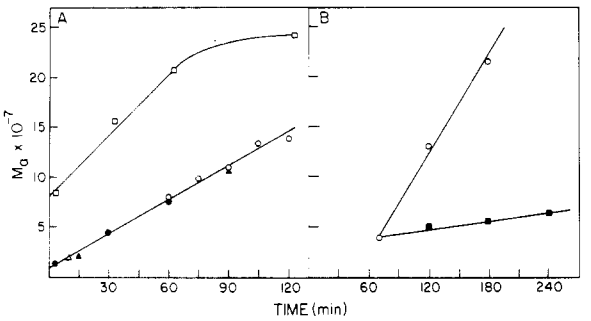

FIGURE 7: DNA chain growth in 10T1/2 cells. The average molecular weights (M_a) of the distributions of nascent DNA were determined after pulse and chase experiments as described under Experimental Procedures. (A) The values of M_a, plotted against total time of DNA synthesis, are shown for DNA from synchronized cells pulsed immediately after being released from the aphidicolin block (lower curve, slope = 1.2×10^6 daltons/min) and compared with those obtained with logarithmically growing cultures (upper curve, slope = 2.1×10^6 daltons/min). Different symbols denote separate experiments. (B) Cells were first released from the aphidicolin block, incubated in fresh medium for 1 h, pulse-labeled for 10 min with [^{3}H]thymidine, and then chased in fresh medium [(○) slope = 1.6×10^6 daltons/min] or in the presence of 2 μg/mL aphidicolin [(■) slope = 1.2×10^5 daltons/min].

periments were performed. Figure 6 shows the distribution profiles of labeled nascent DNA in alkaline sucrose gradients following different times of chase, immediately after the cells were released from the aphidicolin block and pulsed with [^{3}H]thymidine. Very sharp and fairly symmetrical peaks of newly synthesized DNA were observed following a 3-, 10- (Figure 6A), or 15-min (Figure 6B) pulse. There also was a steady increase in molecular weight of the nascent DNA with increasing times of chase in nonradioactive media. In Figure 6C the distribution of nascent DNA labeled by a 15-min pulse with [^{3}H]thymidine and chased for another 165 min is compared to that of parental DNA uniformly labeled with [^{14}C]thymidine. After 3 h of synthesis, a bimodal distribution of the newly replicated DNA was observed (Figure 6C). Approximately 52% of the nascent DNA had already reached the maximum size that can be measured in these gradients, while 48% still lagged behind at about the same position as the DNA molecules pulsed and chased for 2 h (Figure 6B). The molecular weights in the two peak fractions seen in Figure 6C correspond to 1.57×10^8 and 3.48×10^8.

These results show that in cells synchronized by aphidicolin one can follow the growth of nascent DNA in those replicons that initiated synthesis early in S phase. Furthermore, the less heterogeneous size distribution of nascent DNA molecules facilitates the measurements of average molecular weight (M_a). Figure 7A shows the results of plotting M_a vs. time of synthesis of several independent pulse–chase experiments including those in Figure 6. In these synchronized cells, the average molecular weight increased linearly with time for at least 2 h. The slope of the M_a vs. time curve (Figure 7A) expressed in daltons per minute was 1.2×10^6. This provides an estimate of the apparent rate of DNA chain elongation in synchronized C3H 10T1/2 cells. It is actually very close to the average rate of DNA chain growth in a variety of eukaryotic cells, as determined by different procedures [see reviews by Edenberg & Huberman (1975) and Kapp & Painter (1982)]. For comparison, it is also shown in Figure 7A the kinetics of DNA chain growth observed by the same methodology in asynchronous 10T1/2 cells. The slope of the linear part of this curve was estimated as 2.1×10^6 daltons/min. Finally, the effect of aphidicolin on DNA elongation was measured by the experiment shown in Figure 7B. Cells synchronized as described in Figure 2B were released from the aphidicolin block and incubated in fresh medium for 1 h. They were pulse-labeled for 10 min with [^{3}H]thymidine and then chased in fresh medium or in the presence of 2 μg/mL aphidicolin. In this experiment, the rate of DNA chain growth was estimated to be 1.6×10^6 daltons/min in the control cells and 1.2×10^5 daltons/min in the presence of aphidicolin.

Discussion

The results in this paper illustrate the synchronization of C3H 10T1/2 clone 8 cells by the combined use of confluence arrest and inhibition of DNA polymerase α by aphidicolin (Figure 2). This mouse embryo cell line was first selected for its high sensitivity to postconfluence inhibition of cell division (Reznikoff et al., 1973). This characteristic has been used to synchronize 10T1/2 cells without the need for serum deprivation or any other drug treatment (Bertram & Heidelberger, 1974; Grisham et al., 1980). Although this method of synchronization has been quite helpful in studies of cell cycle dependent phenomena (Grisham et al., 1980; Smith et al., 1981), the low rate of entry of cells into S phase makes it less desirable for studies of events occurring early in S phase. In this paper, we have evaluated the usefulness of aphidicolin in accomplishing such a goal.

When C3H 10T1/2 cells were released from contact inhibition and replated in medium containing aphidicolin, they progressed toward S phase at the same rate (8–9% per hour) as control cells but were arrested in early S phase (Figure 3). Once the inhibitor was removed, these cells proceeded through the S phase without any detectable delay (Figures 2 and 3). The 10T1/2 cells were also able to initiate nuclear DNA replication (Figures 3C and 4) in the presence of aphidicolin, and our results essentially agree with those of D'Anna & Tobey (1984). However, cells synchronized and pulse-labeled immediately after the removal of this mycotoxin from the culture medium showed a very symmetrical distribution of labeled nascent DNA at the top of the alkaline sucrose gradients (Figures 5 and 6) and no significant amount of labeling in higher molecular weight DNA. The DNA radiolabeled in the presence of aphidicolin reached the size distribution shown in Figure 4C, which displayed a peak at 1×10^7 daltons. These results are similar to those described by Walters et al. (1976) for the DNA synthesized by Chinese hamster cells entering S phase in the presence of hydroxyurea.

Another important point to consider is the kinetics of inhibition of DNA replication by aphidicolin and its reversibility. As shown in Figure 1, the incorporation of [^{3}H]thymidine in DNA was brought to its minimal level (dependent on the concentration of aphidicolin) in the first few minutes of incubation. Even when the isotope was added to the cells at the same time as the inhibitor (at 2 μg/mL), DNA synthesis in the first 5 min was as low as that detected 1 h later. When aphidicolin was removed from the culture medium, the DNA synthetic activity in the first 15 min was consistently higher than that observed in the subsequent time point (Figure 1). Then it steadily increased and reached control levels 2 h later. However, the number of labeled nuclei observed by autoradiography was practically the same in control and aphidicolin-treated cultures, right after removal of the inhibitor or later (results not shown). These results probably mean that as the concentration of aphidicolin decreased inside each cell the [^{3}H]thymidine incorporation gradually increased. If this interpretation is correct, then the number of replication sites or the rate of elongation (or both) was changing in each cell between the time aphidicolin was removed and 2 h later. It is interesting to note that our kinetic results (Figures 6 and

7A) revealed an apparent constant rate of DNA chain growth between 0 and 120 min after the release of 10T1/2 cells from the aphidicolin block. Therefore, the overall rate of incorporation of [^{3}H]thymidine in DNA in this particular case (Figure 1) was being mainly determined by the number of active replication sites inside each cell.

DNA chain growth is a very complex process, involving first the initiation and bidirectional polymerization of nucleotides followed by ligation of Okazaki fragments in each replicon, merging of adjacent replicons in a cluster, and eventual fusion of independent clusters. This temporal sequence of events could be followed in *Physarum polycephalum* due to the natural, nearly perfect synchrony of DNA replication displayed by this organism (Funderud et al., 1978). As pointed out above, our results (Figure 7A) show a linear growth for 120 min of nascent DNA pulse-labeled at the beginning of the S phase. We observed a discontinuous increase in size of the nascent DNA only between 2 and 3 h as indicated by the bimodal distribution in Figure 6C. Therefore, our results are different from those obtained with primary cultures of mouse embryo cells (Kowalski & Cheevers, 1976) or mouse thymocytes (Lanotte et al., 1977) where nonlinear kinetics of chain growth were observed following shorter chasing times. Also, our data do not reveal any accumulation of long-lived intermediates before 120 min of DNA synthesis, as seen in other systems (Lanotte et al., 1977; Funderud et al., 1978). Recently, Lönn & Lönn (1983) have reported that "aphidicolin inhibits the synthesis and joining of short DNA fragments but not the union of 10-kilobase DNA replication intermediates". Our results seem to indicate an accumulation of DNA intermediates larger than 10 kilobases (kb) and practically no elongation to high molecular weight DNA in the presence of aphidicolin (Figure 4). Figure 7B demonstrates that aphidicolin was an effective inhibitor of DNA chain growth in 10T1/2 cells. Also, the kinetics of inhibition of DNA replication by aphidicolin in the human melanoma cell line used by Lönn & Lönn (1983) was drastically different from our results with 10T1/2 cells (Figure 1). In their experiments, 10 μg/mL aphidicolin was necessary to gradually inhibit the incorporation of [^{3}H]thymidine in DNA to less than 20% in 1 h. They actually reported very little inhibition in the first 15 min of incubation with aphidicolin.

It must be emphasized here that different cell systems respond differently to the inhibitory effects of aphidicolin. It is believed that aphidicolin inhibits DNA polymerase α by interfering with the interactions of this enzyme with dNTPs [see review by Huberman (1981)]. Thus, the size of dNTP pools and the amount of DNA polymerase α and its sensitivity or resistance to aphidicolin ultimately determine the cellular response to this inhibitor (Ayusawa et al., 1981; Sugino & Nakayama, 1980; Tanabe et al., 1983). Furthermore, previous manipulations of the same cell system can modify the final results. For instance, the replication of SV40 chromosomes is severely impaired by the exposure of infected cells to aphidicolin. However, actively replicating SV40 chromosomes seem to be more readily inactivated by aphidicolin than those newly initiated (Dinter-Gottlieb & Kaufmann, 1982, 1983). Thus, SV40-tsA replicons, synchronized at the origin by temperature restriction, could be initiated in the presence of aphidicolin when incubated at the permissive temperature. Upon removal of the drug, the initiated replicons could be elongated and chased to SV40 form I DNA almost to the same extent as seen in control cells (Dinter-Gottlieb & Kaufmann, 1982). Our results are consistent with these observations. When 10T1/2 cells were released from confluence arrest in the presence of aphidicolin, they proceeded toward S phase and initiated nuclear DNA synthesis (Figures 3 and 4). Once the drug was removed, the nuclear DNA could be pulsed and chased to high molecular weights (Figures 5 and 6) at normal rates (Figure 7). However, we have not yet investigated the ability of steady-state replicons to be elongated following treatment with aphidicolin and chase in the absence of this inhibitor. We have observed that logarithmically growing cells are apparently more sensitive to prolonged exposures to aphidicolin than cells released from confluence arrest (results not shown). Therefore, our data in 10T1/2 cells are in agreement with the observations of Dinter-Gottlieb & Kaufmann (1982, 1983) in SV40 and support their conclusion that replicon activation can be uncoupled from DNA chain elongation by treatment of prereplicative cells with aphidicolin. Whether this is due to the participation in the initiation process of a different polymerase or a form of DNA polymerase α resistant to aphidicolin (Tanabe et al., 1983) still remains to be investigated.

The apparent rate of chain growth estimated from the data in Figure 7A (1.2 μm/min) indicates that nascent DNA approaching 140 μm in length could be synthesized in 10T1/2 cells in the first 2 h of the S phase (see also Figures 5 and 6). The average replicon size in 10T1/2 cells has not been determined, but values ranging from 30 to 250 μm have been described in other mouse cell lines [see review by Edenberg & Huberman (1975)]. At the present time, we do not know whether our results reflect activation of very long replicons at the beginning of S phase or the continuous joining of asynchronously initiating, very small replicons inside a cluster. Taylor and co-workers have proposed a 4-μm periodicity in the distribution of potential replicon initiation origins in Chinese hamster ovary cells (Taylor & Hozier, 1976). The number of origins actually used for initiation in each S phase would be only 1 in 15–20 potential origins, but this ratio increased when cells were held at the beginning of S phase by fluorodeoxyuridine inhibition of DNA synthesis (Taylor, 1977). However, in this same system, the average length of nascent DNA strands was seen to increase linearly as a function of the labeling time for a period of 20 min without any clear periodicity (Laughlin & Taylor, 1979). It is an open question whether the above observations are peculiar to Chinese hamster ovary cells synchronized with fluorodeoxyuridine or whether they are also relevant to this study with C3H 10T1/2 cells synchronized with aphidicolin. However, it is clear from our data in Figure 7 that the apparent rate of chain elongation measured by pulse and chase experiments is slightly higher when synchronized cells are used at 1 h after the release from the aphidicolin block (Figure 7B) and much higher in asynchronous populations (Figure 7A). In our opinion, these differences do not reflect faster rates of DNA elongation but rather the complexity of DNA chain growth in eukaryotes, which is also affected by replicon merging.

Finally, our results indicate that aphidicolin is a useful tool for synchronizing cells but it is not necessarily better or worse than other DNA synthesis inhibitors, such as hydroxyurea, fluorodeoxyuridine, or methotrexate, in arresting cells at the beginning of the S phase. All of these agents, regardless of their mechanism of action, inhibit DNA replication after it has begun and cannot prevent S-phase initiation [see review by Hochhauser et al. (1981)]. Furthermore, the arrest of cells in early S phase by any of these DNA synthesis inhibitors seems to trigger other cellular events that should not be overlooked. For example, they may all induce changes in histone H1 content and chromatin structure (D'Anna & To-

bey, 1984), increase the number of initiation sites that are activated in early S phase (Taylor, 1977), and lead to gene amplification [Huang et al., 1983; Mariani & Schimke, 1984; see review by Hamlin et al. (1984)]. Most of these observations were made in Chinese hamster cells, and we do not know whether the synchronization protocol described in this paper also triggers these same responses in 10T1/2 cells. Nonetheless, since the induction of synchrony is a necessary task in many biochemical experiments requiring large numbers of cells, the choice of agents and protocols should rely on the particular cellular system in use and its sensitivity to the inhibitor. Furthermore, investigators should always keep in mind that perturbations of cellular physiology may be unavoidable in any synchronization protocol and these should be taken into account when experimental results are interpreted.

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