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Synchronization of HeLa cell cultures by inhibition of DNA polymerase α with aphidicolin

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ABSTRACT

Both the inhibitory effect of aphidicolin on the replicative α-polymerase and the reversibility of its action in vivo (Pedrali-Noy & Spadari, 1979, Biochem. Biophys. Res. Commun. 88, 1194-2002) allow the synchronization of cells in culture. Aphidicolin prevents G_1 cells from entering the DNA synthetic period, blocks cells in "S" phase, allows G_2, M and G_1 cells to continue the cell cycle and to accumulate at the G_1/S border. Aphidicolin is a more useful reagent than hydroxyurea and thymidine because it does not affect cell viability or "S" phase duration and does not interfere with the synthesis of dNTPs or DNA polymerases. In fact cells exposed to the drug continue to synthesize all three DNA polymerases α, β and γ as well as all dNTPs which, when the block is removed, are present at levels optimal for DNA initation and replication. The technique is simple amd can be applied to cells growing in suspension or monolayers and allows one to harvest large quantities of synchronized cells.

INTRODUCTION

Hydroxyurea and thymidine are two compounds very often employed to induce partial synchrony at the G_1/S boundary. Their use, however, is not without problems. Hydroxyurea at low concentrations does not lead to uniform synchrony at the G_1/S boundary and both high concentrations and increased duration of exposure are toxic to "S" phase cells (2, 3). Thymidine block also does not completely arrest cells at the G_1/S interphase (4, 5) and it induces both chromosome aberrations (6) and cell-cycle dependent alterations in metabolism (7, 8).

For the reasons cited above and because nucleoside diphosphate reductase seems to be the common target for both hydroxyurea and thymidine (9, 10), it is highly desirable to find another inhibitor lacking the side effects just mentioned and acting on another step of DNA replication. The inhibitory action (1, 11, 12) of aphidicolin on replicative (13-16) DNA polymerase α together with its reversibility of action (1) suggest it would be valuable for inducing synchrony. Indeed from this study it appears that

aphidicolin blocks cells in "S" phase by inhibiting the replicative DNA polymerase α and allows G_2, M and G_1 cells to accumulate truly at the G_1/S border providing cells which are useful for studies of biochemical events within the mammalian cell cycle. The drug does not seem to affect cell viability, "S" phase duration, and does not interfere with the synthesis of the four deoxynucleoside triphosphates and DNA polymerases α, β and γ.

MATERIALS AND METHODS

Chemicals: Unlabeled deoxyribonucleoside triphosphates, poly[d(A-T)], poly[d(I-C)] and E. coli DNA polymerase I (Klenow fragment) were purchased from Boehringer, Mannheim, W.Germany. Dithiothreitol (DTT) was from Miles Laboratory, Inc., Kankakee, Ill., USA. Bovine serum albumin (BSA) A grade was from Calbiochem., Lucerne, Switzerland. [^{3}H] deoxyribonucleoside triphosphates and [Me-^{3}H]thymidine were from Radiochemical Center. Poly(A) and oligo(dT)$_{12-18}$ were from PL-Biochemicals, Milwaukee, Wis., USA.

Preparation of templates: Activated calf thymus DNA was prepared as decribed by Pedrali-Noy and Weissbach (17).

Cell culture: Growth of HeLa cells in spinner cultures has been described previously (18).

Laser flow cytofluorometric analysis: 10^6 HeLa cells were suspended in 1 ml of fluorescent staining solution (19). The stained samples were kept at 4°C and analyzed within the next two to three days in a Bio-Physics (now Ortho Instruments) Cytofluorograph model FC 200/4800 A equipped with a 100 mW argon ion laser. The DNA histograms representing the fluorescence intensity distribution among cells indicate the number of cells per channel in the ordinate and the relative fluorescence intensity which varies in proportion with the DNA content in the abscissa. To facilitate the comparison between different samples in a given figure, counting was normalized to a predetermined peak height of 1,600.

Measurement of radioactivity: Synthesis of DNA troughout the growth cycle was measured by the uptake of [^{3}H]thymidine during a 30 min pulse at 37°C at predetermined times (1).

Preparation of extracts: Samples (approximately 6x 10^6 cells) were suspended in 200 µl of 10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM dithiothreitol. The suspension was then made 0.1 M KCl, 0.1 M potassium phosphate (pH 7.5), 0.5% Triton-X-100 and sonicated 2x 5 sec with the microtip of a Branson Sonifier at a setting of 50 W. This extract was used to determine the activity of DNA polymerases. Protein determinations

were carried out as described (20).

Assay of DNA polymerases: Each reaction (0.1 ml) was carried out at 37°C. The α-polymerase was assayed in 20 mM potassium phosphate (pH 7.2), 0.5 mM DTT, 7 mM $MgCl_2$, 150 µg/ml BSA, 200 µg/ml of activated DNA, 100 µM dGTP, dCTP,dATP, and 50 µM [^{3}H]dTTP (500 cpm/pmol).

Since the ß-polymerase partially responds to the α-polymerase assay (14) the α-polymerase data presented here have been corrected for the contribution on the ß-enzyme evaluated by the addition of N-ethylmaleimide (NEM) in a parallel DNA polymerase α assay (14).

The ß-polymerase reaction was carried out in 50 mM Tris-HCl (pH 8.5), 0.1 M KCl, 7 mM $MgCl_2$, 0.5 mM DTT, 150 µg/ml BSA, 200 µg/ml of activated calf thymus DNA, 100 µM dGTP,dCPT,dATP and 50 µM [^{3}H]dTTP (500 cpm/pmol). When the enzyme samples are preincubated with 10 mM NEM at 0° for 30 min (to inactivate DNA polymerases α and γ) the ß-assay is strictly specific for the ß-polymerase (14). DNA polymerase γ was assayed according to Knopf et al. (21). The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 50 mM potassium phosphate (pH 8.5), 0.13 M KCl, 0.5 mM $MnCl_2$, 1 mM DTT, 150 µg/ml BSA, 50 µg/ml poly(A):oligo$(dT)_{12-18}$ (at a ratio of 5:1 by weight) and 50 µM [^{3}H]dTTP (500 cpm/pmol). Under these conditions the assay is strictly specific for the γ-polymerase due to the fact that ß-polymerase activity in the presence of poly(A):oligo(dT) is inhibited by phosphate (21) and α-polymerase does not utilize this polynucleotide as template primer (14).

A unit of DNA polymerase is defined as one nmol of total deoxynucleotide incorporation into acid insoluble form in 60 min at 37°C.

Extraction and determination of deoxyribonucleoside triphosphates: Approximately 1 to 2 x 10^7 HeLa cells were extracted with 60% ethanol for 10 min at 30°C (22). The dNTPs were determined as described by Skoog (23). In a final volume of 0.1 ml each assay contained: 50 mM Tris-HCl buffer, pH 8.2, 10 mM $MgCl_2$, 1 µg poly[d(I-C)] or poly[d(A-T)], 10 µM [^{3}H]-labeled deoxyribonucleoside triphosphate (spec. activity about 1 Ci/mmol), 0.25 units of the Klenow fragment of DNA polymerase I (E. coli) and 10-70 pmol of one limiting unlabeled deoxyribonucleoside triphosphate or an aliquot of the cell extract. Incubation was for 40 min at 37°C.

RESULTS

Synchronization of HeLa cells with aphidicolin: We have recently suggested (1) that the inhibitory action of aphidicolin on the replicative DNA polymerase α together with its reversibility of action could make it a

valuable reagent for synchronization of cell cultures. Here we show that aphidicolin accumulates cells at the G_1/S border thus allowing a simple and rapid synchronization of viable cells.

Figure 1A is a DNA histogram of asynchronously growing HeLa cells (control): the majority (60%) of the cells are in G_1 (peak at channel number 19). They are separated from cells having twice this amount of DNA (G_2 and M cells - approximately 20% - at peak channel 38) by "S" phase cells which are characterized by an intermediate amount of DNA with a mean distribution at approximately channel number 28.

The DNA histograms are not altered 30 min after addition of aphidicolin (Fig. 1B). After 7 h (Fig. 1C) and especially after 24 h (Fig. 1D) the number of cells still in "S" remains constant whereas most of those in G_2 and M have reached G_1. This appears to be due to the selective inhibition of the replicative DNA polymerase α by aphidicolin (1, 11, 12, 24). Following addition of the drug, the rate of DNA synthesis as measured by [^{3}H]thymidine uptake has dropped to 2% of its original value (see below) and remains at this low level for the next 24 h (this residual DNA synthesis is probably

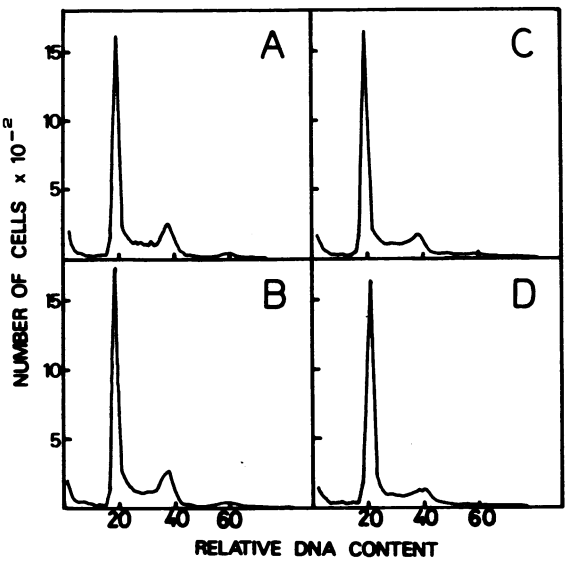

Figure 1. Cell cycle kinetics analysis of HeLa cells exposed to aphidicolin. Hela cells growing in suspension at a density of 9 x 10^5/ml were exposed to 5 µg/ml aphidicolin. At 0' (A), 30' (B), 7 h (C) and 24 h (D), 10 ml aliquots were taken from the stock culture. One ml was used for DNA histograms and 9 ml for the experiments described in Figure 3.

mitochondrial DNA synthesized by DNA polymerase γ which is resistant to aphidicolin; Pedrali, Hardt & Spadari, unpublished). A time period of 24 h was chosen because it is greater than the length of G_2+M+G_1 phases and because the inhibition of cellular DNA synthesis by aphidicolin is still reversible after 24 h. Shorter exposure (18-20 h) to aphidicolin gave similar results.

The chronology of "S" phase traverse by aphidicolin treated HeLa cells, allowed to resume growth in fresh medium, is shown in Figure 2. Ninety min after reversal (Fig. 1A) the cells have progressed some four channels towards the "S" region of the DNA histogram. Channel 28 situated halfway through "S" was reached at 4 h 30 (Fig. 2B). "S" traverse was practically completed within 8 h after resuspension in fresh medium (Fig. 2D). [^{3}H]-thymidine uptake closely follows flow cytofluorometry analysis (Fig. 3B). Two points are worth mentioning: first, a rapid increase in the rate of thymidine incorporation occurs at 90 min, secondly, the maximal rate of DNA synthesis occurs between 4 and 6 h corresponding to the flow cytofluorometry analysis which indicates the wave of synchronized cells is half-

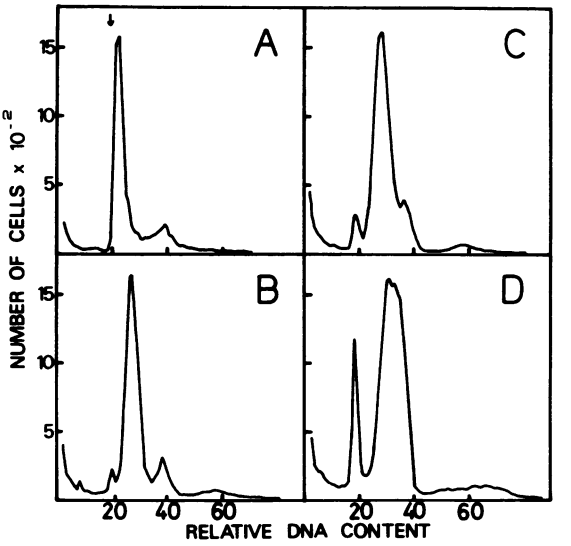

Figure 2. Cell cycle kinetics analysis of HeLa cells exposed to aphidicolin. HeLa cells maintained 24 h in presence of aphidicolin (Figure 1) were washed three times and allowed to resume growth in fresh pre-warmed culture medium. DNA histograms were made at 1 h 30' (A), 4 h 30' (B), 6 h (C) and 8 h (D) after reversal.

way through "S" (Fig. 2B).

In addition, following aphidicolin release the number of cells remains relatively constant as it varies from 9 x 10^5/ml to 9.3 x 10^5/ml within the first 10 h when synthesis of DNA is completed indicating the high synchronization achieved with a single exposure to aphidicolin. Shortly there after the cells divide in synchrony and double providing further proof that they had accumulated at the G_1/S border during exposure to aphidicolin and indicating the absence of toxic effects induced by the drug. Thus, exposure to aphidicolin does not affect the capacity for cycle traverse upon subsequent removal of the drug.

In contrast to synchronization experiments utilizing thymidine or hydroxyurea alone, nearly all cells are recruited to synchronously traverse "S" phase. The few cells which remain trailing behind the "S" wave (Fig. 2C) can be forced to accompany the main stream by a second 12 h exposure to aphidicolin (data not shown).

Effects of aphidicolin on DNA polymerase α, ß and γ activities, protein synthesis and rate of DNA synthesis during cycle traverse: The inhibition of DNA synthesis (25) by aphidicolin is mediated by the inhibitory effect of this drug on DNA polymerase α (1, 11, 12, 24). We wondered whether the interaction of the drug with the enzyme might affect also its de novo synthesis, either by interfering negatively with its production or by causing an overproduction in response to the low availability of the replicative enzyme in the cell. Thus, we have followed the level of DNA polymerase α in the cell during DNA replication block due to aphidicolin, as well as the levels of the ß and γ polymerase, which, not being involved in chromosomal DNA replication (15, 16), may be considered as an internal standard with which to compare the possible variation in α-polymerase levels.

Figure 3A shows that the levels of all three DNA polymerase increase (in parallel with total protein synthesis - Fig. 3B) during incubation of cell cultures with aphidicolin and their activity is not dependent on DNA synthesis which is immediately blocked upon addition of the drug (Fig. 3B). After a 24 h exposure to aphidicolin, the levels of all three DNA polymerases are sufficient to allow DNA replication and subsequent cell duplication upon reversal of the block.

Effect of aphidicolin on the synthesis of deoxyribonucleoside triphosphates: Both thymidine and hydroxyurea block DNA synthesis by reducing the level of dNTPs. In order to determine if aphidicolin also had a similar effect on the dNTP pool we measured dNTP levels during exposure to

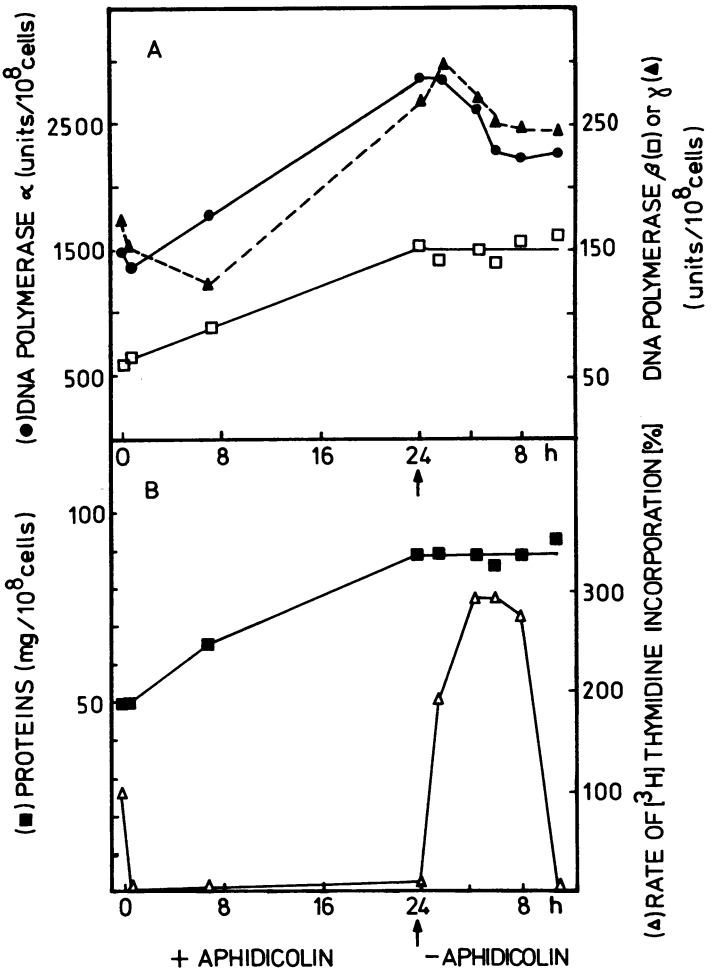

Figure 3. Effect of aphidicolin on DNA polymerase α, ß, γ activities (A), protein synthesis, and rate of [^{3}H] thymidine incorporation (B). HeLa cells maintained 24 h in presence of 5 µg/ml aphidicolin were allowed to resume growth in fresh medium. At predetermined times during the experiments described in Figure 1 and 2, 10 ml aliquots were taken from the stock culture. One ml was incubated in duplicate with [^{3}H] thymidine at 2 µC/ml for 30 min to monitor the rate of DNA synthesis (Figure 3 B). 7 ml were centrifuged twice, the cells being suspended each time in 40 ml of ice cold growth medium lacking serum. The cell pellet was then resuspended in the same volume of ice cold phosphate buffer saline, centrifuged again and the cells were stored at -90°C prior to the determination of DNA polymerase α, ß and γ (3 A) as described in Materials and Methods. Crude cell extracts were used also for protein determination (3 B). The variance for each determination of DNA polymerase α, ß and γ was less than 5, 15 and 12 % respectively. The variance for protein determinations was less than 10 %.

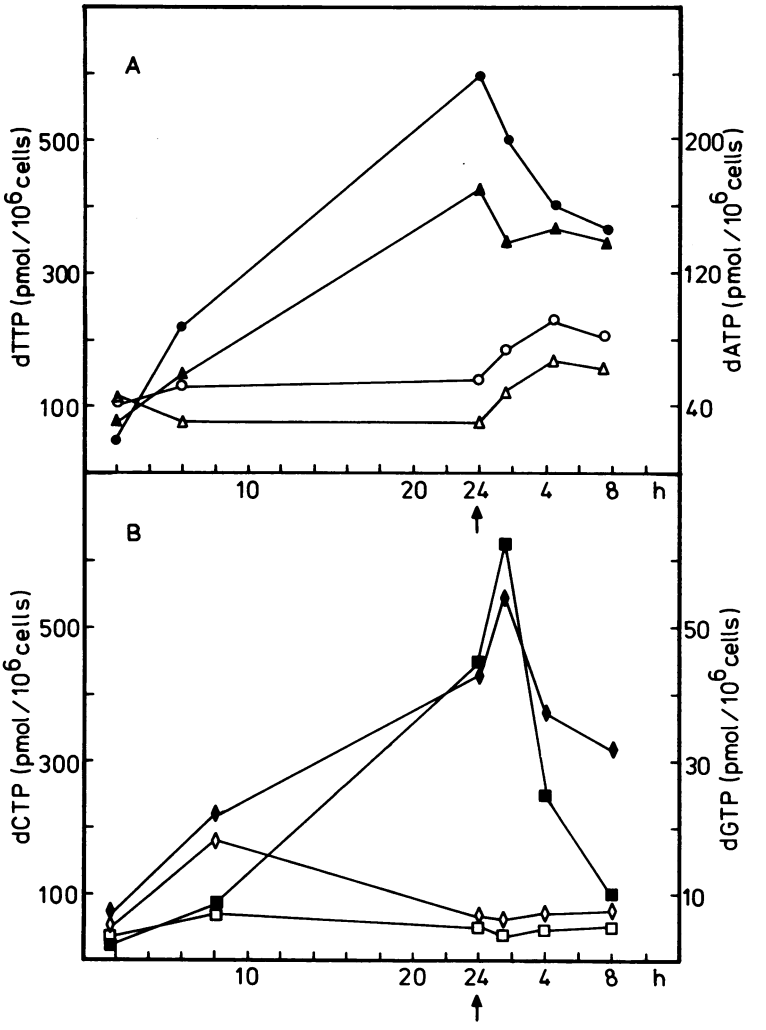

Figure 4. Effect of aphidicolin on deoxyribonucleoside triphosphate pool. HeLa cells grown in suspension (4 x 10^5 cells/ml) were incubated in the absence (control) or in the presence of 5 µg/ml aphidicolin for 24 h. The cells from both cultures were then sedimented, washed, suspended in fresh prewarmed culture medium and then incubated at 37°C. At the indicated times 25 ml aliquots were removed from both cultures for the determination of the four dNTP pools. The open symbols indicate levels of dNTPs in control cells; the filled symbols indicate levels of dNTPs in aphidicolin-treated cells.

A: ▲ , △ dATP; ● , ○ dTTP.
B: ◆ , ◇ dGTP; ■ , □ dCTP.

aphidicolin. Figure 4 indicates that the levels of all four dNTPs continue to increase during exposure to aphidicolin. Upon removal of the drug the levels rapidly decrease as the dNTPs are utilized for DNA synthesis. Analysis of control cultures, where DNA synthesis is continuing, indicates that the levels of the four dNTPs remain constant troughout the incubation period and increase slightly upon transfer to fresh medium.

DISCUSSION

Analysis by laser flow cytofluorometry of the effects produced by aphidicolin on the cell cycle traverse of HeLa cells growing in suspension corresponds directly to the variations in the rate of DNA synthesis as obtained by radioactivity measurements. Using cytofluorometry the readily reversible action as well as the ability of aphidicolin to synchronize HeLa cells can easily be shown. The results presented here indicate that aphidicolin is as good if not better than hydroxyurea or thymidine for synchronizing tissue culture cells. One definite advantage over hydroxyurea or thymidine is that aphidicolin accumulates cells at the G_1/S boundary. Following a single exposure to aphidicolin the block can be removed and almost all the cells are recruited to synchronously traverse "S" phase and subsequently all divide.

Because of the high rate of synchrony decay during G_1 period of the cell cycle (26) it is often desirable and necessary, in the study of late interphase events, to resynchronize cells initially synchronized by mitotic selection or by isoleucine deprivation. Hydroxyurea, which has often been used for this purpose, at low concentrations does not prevent synchronized G_1 cells from entering the DNA synthetic period (3) and at high concentrations shows an "S" phase cytotoxicity (2). Aphidicolin permits cells to traverse G_2, M and G_1, but prevents replicative DNA synthesis through the inhibition of the α-polymerase. Thus aphidicolin permits the isolation of cell populations truly and reversibly arrested at the G_1/S border which are more suitable for detailed studies of events associated with initiation of genome replication in the immediate pre-S phase. The labeling of the replicon's origin and the timing of chromosomes replication could then more carefully be studied in cells synchronized or resynchronized with aphidicolin.

The 24 h treatment with aphidicolin does not affect the synthesis of the four dNTPs and of DNA polymerases. Both all dNTPs and all DNA polymerases (in particular DNA polymerase α) increase troughout the incubation period

and, following removal of the drug, are present at levels optimal for DNA initiation and replication. The inhibition of DNA polymerase α does not in any way interfere with the regulation of its synthesis by the cell and the regulatory apparatus of the cell is apparently insensitive to the inavailability of the replicative enzyme for a 24 h period.

Thus aphidicolin treatment appears to be a suitable method for synchronization of large quantities of suspension and monolayer (Pedrali-Noy and Spadari, unpublished) cell cultures. The drug seems less toxic than the available ones since it does not reduce cell viability and does not interfere with the synthesis of deoxyribonucleoside triphosphates or polymerases required for rapid cell division after reversal of the block.

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