Biochem. J. (1992) 281, 87–93 (Printed in Great Britain)

Deficiencies in DNA replication and cell-cycle progression in polyamine-depleted HeLa cells

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Synchronized HeLa cells depleted of polyamines by α-difluoromethylornithine exhibited substantially decreased DNA synthesis, and proliferation ceased after the release of the cells into S phase. Nuclei from these cells synthesized 70–80% less DNA than did nuclei from control cells. Extraction of isolated nuclei with 0.3 M-KCl decreased DNA synthesis by about 60%, which was recovered almost completely in control cell nuclei by reconstitution with the salt extracts of these nuclei. On the other hand, salt extracts of polyamine-depleted nuclei restored only 50% of DNA synthesis in extracted control nuclei. Salt extracts of control cell nuclei contained twice the DNA polymerase α activity of polyamine-depleted nuclear extracts. Extracts of cell lysates of both control and polyamine-depleted HeLa cells exhibited similar DNA polymerase α activity, suggesting that uptake of the enzyme or its retention by the nuclei of polyamine-depleted cells was decreased. Polyamine-depleted nuclei also showed altered phosphorylation of a 31 kDa protein as compared with control nuclei. Almost normal DNA synthesis, cell proliferation, DNA polymerase α activity and nuclear protein phosphorylation were restored in polyamine-depleted cells grown in medium supplemented with 20 μM-spermidine at least 10–12 h before S phase. Cultures in which proliferation was blocked by α-difluoromethylornithine did not exhibit synchronous growth after the block was removed. Thus it may be concluded that HeLa cells depleted of polyamines are not inhibited at a single control point in the cell cycle, but are arrested at diverse sites throughout G1 phase.

INTRODUCTION

Although their specific physiological roles are still unclear, it has been well established that the polyamines spermine, spermidine and putrescine are essential for mammalian cell DNA synthesis and proliferation. Early studies with bovine lymphocytes (Fillingame *et al.*, 1975; Knutson & Morris, 1978) utilized the inhibitor methylglyoxal bis(guanylhydrazone) (MGBG) to decrease cellular polyamines and to demonstrate inhibition of DNA synthesis in isolated nuclei. The interpretation of these studies was controversial because of the cytotoxicity of MGBG and its apparent lack of specificity as an inhibitor of polyamine biosynthesis. Nevertheless, these experiments stimulated critical investigations of the role of polyamines in DNA synthesis by bovine lymphocytes (Seyfried & Morris, 1979) and HeLa cells (Branca & Herbst, 1980; Herbst & Elliott, 1981; Gallo *et al.*, 1986) utilizing the specific inhibitor of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) α-difluoromethylornithine (DFMO) to decrease cellular polyamines. The polyamine content of bovine lymphocytes in cultures containing DFMO was decreased by approx. 50% and the synthesis of DNA by the lymphocytes, or their isolated nuclei, was decreased comparably. Only traces of putrescine and spermidine, and about 30% of the normal content of spermine, were found in HeLa cells cultured in media containing DFMO, and the synthesis of DNA by S-phase cells from these cultures, or by isolated nuclei from the cells, was inhibited by 70–80%. As reported in experiments with MGBG (Krokan & Eriksen, 1977; Knutson & Morris, 1978), the addition of spermidine to the HeLa-cell cultures containing DFMO reversed the inhibition of DNA synthesis in the cells, or in isolated nuclei (Gallo *et al.*, 1986), but supplements of spermidine to the assays of nuclei *in vitro* did not reverse the inhibition of DNA synthesis (Herbst & Elliott, 1981).

In mammalian cells, the highest ornithine decarboxylase activity was attained during mid-G1 to S phase of the cell cycle (Heby *et al.*, 1976; Seidenfeld *et al.*, 1986), and the complete reversal of the inhibition by DFMO of DNA synthesis in HeLa cells was contingent on the addition of spermidine to the cultures at least 10–12 h before the initiation of S phase (Gallo *et al.*, 1986).

In the present investigation, we have attempted to characterize the deficiencies in nuclei isolated from HeLa cells depleted of polyamines by DFMO. Subnuclear fractions of S-phase nuclei were prepared by salt extraction, and reconstitution of the nuclei was studied by the methods introduced by Brun & Weissbach (1978) and extended by Enomoto *et al.* (1983*a*,*b*). We have observed deficiencies in DNA polymerase α activity contained in the salt extract of nuclei from polyamine-depleted cells, as well as deficiencies in the DNA template of the salt-extracted nuclei of these cells.

By utilizing techniques previously described (Pritchard *et al.*, 1983; Vishwanatha *et al.*, 1986*a*,*b*), DNA polymerase α in nuclei isolated from polyamine-depleted cells was assayed with DNA templates containing low and high base/primer ratios to determine whether the enzyme functioned as a complete holoenzyme. Comparisons were made between the nuclear and total cellular DNA polymerase α activity to determine whether uptake or retention of the enzyme by the nucleus was modified in polyamine-depleted HeLa cells.

The possibility that polyamines may regulate cell-cycle traverse at a single control point was examined by attempting to synchronize HeLa-cell cultures with DFMO. Finally, studies were performed to determine changes in nuclear protein phosphorylation resulting from the depletion of cellular polyamines and interruption of cell cycle traverse.

EXPERIMENTAL

Chemicals

Spermidine hydrochloride was obtained from Calbiochem–Behring Corp. (La Jolla, Ca, U.S.A.). Thymidine was purchased

Abbreviations used: DFMO, α-difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone).

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from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and unlabelled deoxyribonucleoside triphosphates were from Pharmacia P-L Biochemicals (Piscataway, NJ, U.S.A.). Reagents for the preparation of buffers and the components of the assays of DNA synthesis were obtained from Sigma. DFMO was generously provided by Dr. Peter P. McCann, Merrell Dow Research Laboratories, Cincinnati, OH, U.S.A. The [*methyl*-^{3}H]dTTP (10–20 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from NEN Research Products (Billerica, MA, U.S.A.). The chemicals used in the phosphorylation assay and stop solution were purchased from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). 'Activated' calf thymus DNA was purchased from Sigma, calf thymus DNA from Calbiochem-Behring Corp., and SDS from Bio-Rad (Richmond, CA, U.S.A.).

Synchronization and polyamine-depletion of cell cultures

HeLa S_3 cells were obtained from Dr. Sheldon Penman (M.I.T., Cambridge, MA, U.S.A.), and maintained as suspension cultures in Ca^{2+}- and Mg^{2+}-free Basal Modified Eagle's (BME) medium (GIBCO, Grand Island, NY, U.S.A.) as described previously (Gallo *et al.*, 1986). Control cultures, or cultures supplemented with 1 mM-DFMO to obtain cells deficient in polyamines, were synchronized for S-phase DNA synthesis by the double-thymidine-block procedure described by Gallo *et al.* (1986). The cultures were harvested by centrifugation (500 *g*, 10 min at 25 °C) and the cell pellets were washed by two cycles of resuspension in serum-free BME medium to remove excess thymidine. The washed cells were resuspended in BME medium (control cultures) or in BME medium containing 1 mM-DFMO (polyamine-depleted cultures) at 5×10^5 cells/ml and incubated to initiate S-phase DNA synthesis. In some experiments, as noted, cell viability was monitored by microscopically examining a sample of cell suspension stained with Erythrosin B [0.08% (w/v) in 0.4% NaCl]. Cell proliferation was determined by diluting samples of cell cultures with saline and counting cell numbers with a Royco automatic cell counter.

Preparation of cell nuclei: salt extraction and reconstitution of nuclei

Synchronized cells at 3 h after initiation of S phase were centrifuged and nuclei isolated by our modification ('buffer A' procedure) of the protocol developed by Krokan *et al.* (1975) as described below.

A cell pellet containing 50×10^6 cells was resuspended in 10 ml of cold buffer A (10 mM-Hepes, 2 mM-EGTA, 3 mM-$MgCl_2$, 2 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride, pH 7.5 at 4 °C) and centrifuged (1000 *g*, 5 min at 4 °C). The cell pellet was resuspended in 1 ml of buffer A containing 0.025% Triton X-100 and transferred to a 7 ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ, U.S.A.) in an ice bath and incubated in ice for 7 min. The cell suspension was homogenized by 20 strokes of the Dounce pestle, after which 1 ml of buffer A containing 0.075% Triton X-100 was added. The lysate was transferred to a 13 ml Corex tube and centrifuged (1000 *g*, 5 min at 4 °C). The supernatant was decanted and the pellet of nuclei was resuspended in buffer A (10×10^6 cell equivalents/ml; 4 °C). The nuclei in the suspension were counted in a haemocytometer after dilution in 0.08% Erythrosin B/0.4% NaCl solution. Lysis of the cells was complete, i.e. whole cells excluding the dye were not present and cell fragments were absent from the suspension of intact stained nuclei. For the preparation of cell lysates, from which both nuclei and cytosol were extracted for the assay of DNA polymerase α (see below), the cell lysis was modified by eliminating Triton X-100 and homogenizing by 30 pestle strokes to achieve complete cell rupture.

For the assay of DNA synthesis by intact nuclei, samples of the nuclear suspension in buffer A containing 2.5×10^6 nuclei were transferred to 13 ml Corex tubes in an ice bath. After centrifugation (1000 *g*, 5 min at 4 °C), the small nuclear pellet was resuspended and the components of the assay for DNA synthesis (see below) were added to the tubes held in the ice bath.

Salt-extracted nuclei were prepared by resuspending a pellet containing 100×10^6 nuclei in 1 ml of buffer A containing 0.3 M-KCl and incubating the suspension in an ice bath for 30 min with occasional gentle shaking. The suspension was centrifuged (1000 *g*, 5 min at 4 °C) and the salt extract decanted into a small Corex tube in an ice bath. The pellet of salt-extracted nuclei was resuspended in 2 ml of cold buffer A (50×10^6 nuclei/ml) and held on ice. Samples (50 μl), containing 2.5×10^6 salt-extracted nuclei, were pipetted into 13 ml Corex test tubes and the components of the assay for DNA synthesis (see below) were added to the tubes in an ice bath. 'Reconstitution' of the salt-extracted nuclei was achieved by addition of portions of the 0.3 M-KCl extract of the nuclei to the assay tubes containing the nuclei.

Assay for DNA synthesis

Assay components were added to the Corex assay tubes, containing suspensions of either intact nuclei or salt-extracted nuclei and salt extracts of nuclei, in a final volume of 0.25 ml as follows: 50 mM-Tris/HCl (pH 7.5, 37 °C), 8 mM-$MgCl_2$, 6.5 mM-ATP, 2 mM-dithiothreitol, 90 mM-KCl (including the KCl contained in the salt extracts), 100 μM (each) of dATP, dCTP, dGTP, dTTP and 2 μCi of [*methyl*-^{3}H]dTTP. In all experimental protocols, four replicate assay tubes were prepared, three of which were incubated for 30 min at 37 °C and one of which was held on ice as an unincubated blank. After 30 min, all tubes were transferred to the ice bath. The nuclei in each assay tube were collected on 2.4 cm Whatman GFC glass-fibre filters on a 12-place manifold (Millipore, Bedford, MA, U.S.A.) under low vacuum, and the nuclei on the filter were washed with 2×3 ml of cold buffer A. Cold 4% (v/v) $HClO_4$ (15–20 ml) was added to each filter well of the manifold, and the acid solution was slowly filtered by gravity through the nuclear pellet on the filter. The resulting precipitate on the filter was washed with 15–20 ml of cold 80% ethanol, followed by 15–20 ml of cold ethanol under low vacuum. The filters were transferred to scintillation vials, dried under a heat lamp, and 10 ml of scintillation fluid [0.4% 2,5-diphenyloxazole (PPO) and 0.008% *p*-bis-*o*-methylstyrylbenzene (bis-MSB) in toluene] was added. Sample vials were counted for radioactivity in a Beckman model LS-7000 spectrometer.

Determination of DNA polymerase α activity

This was done in salt extracts of either nuclei or cell lysates by the procedure of Enomoto *et al.* (1983*b*), which was optimized for the assay of DNA polymerase α. Samples of either nuclear or cell-lysate salt extracts (10 μl) containing 1×10^6 nuclear or cellular equivalents were pipetted into four replicate assay tubes (in an ice bath), to which were added the following components in a final volume of 50 μl: 50 mM-Tris/HCl (pH 7.5, 37 °C), 2.5 mM-$MgCl_2$, 2 mM-dithiothreitol, 10 μg of bovine serum albumin, 100 μM each of dATP, dCTP and dGTP, and 20 μM-dTTP, 10 μg of 'activated' calf thymus DNA and 0.5 μCi of [*methyl*-^{3}H]dTTP. In some assays (see below) 'activated' calf thymus DNA was replaced with heat-denatured DNA prepared by the method described by Baril *et al.* (1977). Three replicate assay tubes were incubated at 37 °C for 30 min, and one replicate was held on ice as an unincubated blank. After the incubation period, the contents of each assay tube were transferred with Pasteur pipettes to 0.75 in (2 cm) squares of Whatman no. 1 filter paper, and the papers were placed in a beaker of 5% (v/v) trichloroacetic

acid (10 ml/filter-paper square) in an ice bath and held in this solution for 10 min with occasional swirling. The liquid was removed by aspiration and the paper squares were washed in the beaker with 2 × 0.5 vol. of cold trichloroacetic acid, followed by aspiration of the wash solution. After a final wash with 1 vol. of cold 95% ethanol, the paper squares were placed in scintillation vials, dried under a heat lamp and counted for radioactivity in PPO/bis-MSB in toluene in a Beckman model LS-7000 spectrometer.

Analysis of cells and nuclei for polyamines

Washed cell pellets [(12.5–25) × 10^6 cells] from synchronized cultures, or nuclei isolated from homogenates of comparable numbers of cells, were resuspended in 0.5 ml of cold 0.2 M-$HClO_4$ and kept overnight at 4 °C. After centrifugation (1000 *g*, 5 min at 4 °C) the $HClO_4$ supernatant was removed. The analysis of polyamines was performed on the $HClO_4$ extracts of cells or nuclei by the modification of Seiler's dansyl procedure as previously described (Herbst & Dion, 1970).

Phosphorylation of HeLa-cell nuclear proteins *in vitro*

This was studied by procedures developed by Verma & Chen (1986). Samples (100 μl) of the buffer A suspension (approx. 10 × 10^6 nuclei) were added to 1.5 ml Eppendorf tubes, followed by 50 μl of phosphorylation assay mixture containing 3 mM-EGTA, 150 mM-KF, 30 mM-magnesium acetate, 3 mM-sodium molybdate, 1 mM-phenylmethanesulphonyl fluoride and 15 μM-[γ-^{32}P]ATP (3.1 × 10^7 c.p.m./nmol) and incubated for 30 min at 25 °C. The reaction was terminated by addition of 30 μl of 'stop solution' [12% (w/v) SDS, 0.5 M-Tris/HCl, pH 9.0, 5 mM-EDTA, 25% (w/v) sucrose, 10% (v/v) β-mercaptoethanol and 0.1% (w/v) Bromophenol Blue] to each assay tube, followed by boiling for 3 min. Samples of phosphorylation assay mixture and Bio-Rad low-molecular-mass protein standards were applied to a 1 mm-cross-section 15%-acrylamide/SDS slab gel and analysed by discontinuous SDS/PAGE as described by Laemmli (1970). After electrophoresis, the gel was stained with Coomassie Blue (0.25% in 10% acetic acid/50% methanol in water), vacuum-dried on to Whatman filter paper, and exposed to Kodak X-omat AR film (Eastman–Kodak Co., Rochester, NY, U.S.A.).

RESULTS AND DISCUSSION

Depletion of polyamines in HeLa cells and nuclei by DFMO

The effectiveness of DFMO as an inhibitor of polyamine biosynthesis in HeLa cells is illustrated by the substantial decrease in polyamines in cells obtained from cultures synchronized in the presence of the inhibitor (Table 1). Losses of polyamines from nuclei are likely during detergent treatment (0.05% Triton X-100) and washing of nuclei during isolation procedures. Thus the differences in the polyamine contents of control and DFMO-treated nuclei are only indicative of the polyamines retained by the isolated nuclei utilized in this study. It is of interest, however, that control nuclei contain more than one-third of the total cellular spermidine. On the other hand, cells grown in the presence of DFMO contain no spermidine or putrescine. Thus the nuclei of intact DFMO-treated cells would have access to cellular polyamine pools lacking spermidine and putrescine and having severely decreased concentrations of spermine.

Synthesis of DNA by S-phase HeLa-cell nuclei

Nuclei isolated from HeLa cells, synchronized for S phase by a double thymidine block, synthesize DNA *in vitro* comparably with whole cells (Brun & Weissbach, 1978; Herbst & Elliott, 1981). DNA synthesis is inhibited in nuclei isolated from HeLa cells in which polyamines have been depleted by the addition of DFMO to the cultures during the synchronization procedure (Herbst & Elliott, 1981). Reversal of the inhibition of DNA synthesis resulting from the depletion of polyamines occurs in cells, or nuclei derived from them, when polyamines are added to the cell culture 10–24 h before the assay of DNA synthesis (Krokan & Eriksen, 1977; Herbst & Elliott, 1981; Gallo *et al.*, 1986). On the other hand, polyamines added directly to assays of nuclei from polyamine-depleted cells do not increase DNA synthesis *in vitro* (Krokan & Eriksen, 1977; Herbst & Elliott, 1981).

The synthesis of DNA by nuclei isolated during this investigation by our modified 'buffer A' procedure is summarized in Table 2. Nuclei are inhibited by about 75% in DNA synthesis when isolated from cells depleted of polyamines by DFMO. The reversal of the effect of polyamine depletion on DNA synthesis by exogenous spermidine is not complete, but is substantial in nuclei from cells supplemented with the polyamine 10 h before the initiation of S phase.

Table 1. Polyamine contents of HeLa cells and nuclei

HeLa cells were synchronized for S-phase DNA synthesis by the double-thymidine-block procedure. DFMO (1 mM) was present in the medium of DFMO-treated cell cultures throughout the synchronization procedure. Cells were obtained by centrifugation at 3 h after initiation of S phase and nuclei were prepared. Cells and nuclei were analysed for polyamines as described in the Experimental section. The polyamine data are means ± S.E.M. of 3–6 replicate analyses: ND, not detected.

	Content (nmol/10^6 cells or nuclei)	
Polyamine	Control cells	Control nuclei
Spermine	3.3 ± 0.18	0.7 ± 0.05
Spermidine	3.0 ± 0.15	1.1 ± 0.08
Putrescine	1.5 ± 0.14	ND
	'DFMO cells'	'DFMO nuclei'
Spermine	1.3 ± 0.15	0.6 ± 0.06
Spermidine	ND	ND
Putrescine	ND	ND

Table 2. DFMO-induced inhibition of DNA synthesis by HeLa-cell nuclei: partial reversal by the addition of spermidine to the DFMO-containing cell cultures

Nuclei were isolated by the 'buffer A' protocol, 3 h after S phase was initiated in HeLa cells synchronized in the absence (Control) or the presence of 1 mM-DFMO (DFMO) or in the presence of 1 mM-DFMO and 20 μM-spermidine (DFMO/spermidine) added to the culture 10 h before the initiation of S phase. The values are the means of assay results in triplicate samples of nuclei. Variability of assay results between individual samples was less than ± 10%.

Source of nuclei	[^{3}H]TMP incorporated into DNA (pmol/10^6 nuclei)		
	Expt. 1	Expt. 2	Expt. 3
Control	17	9.6	13
DFMO	4.0	2.1	3.1
DFMO/ spermidine	10	6.1	9.4

Table 3. DNA synthesis in HeLa-cell nuclei reconstituted after salt extraction

Nuclei were isolated 3 h after S phase was initiated in cells synchronized in the absence (C) or in the presence of 1 mM-DFMO (D). Intact nuclei (C or D), salt-extracted nuclei (SEN) and SEN reconstituted with 3 nuclear equivalents of salt extract were assayed for [^{3}H]TMP incorporation into DNA. The values are the means ± S.E.M. of 5 separate experiments, each of which contained triplicate samples in each experimental group (numbers in parentheses are percentages of activity with intact control nuclei = 100%).

Source of nuclei	[^{3}H]TMP incorporated into DNA (pmol/10^6 nuclei)
Intact control nuclei (C)	17 ± 1.6 (100)
Intact 'DFMO nuclei' (D)	3.5 ± 1.4 (20)
CSEN	7.0 ± 1.5 (40)
DSEN	1.7 ± 0.5 (10)
CSEN + 3 × C salt extract	13 ± 1.5 (77)
CSEN + 3 × D salt extract	8.3 ± 1.2 (50)
DSEN + 3 × C salt extract	3.6 ± 1.0 (21)
DSEN + 3 × D salt extract	2.5 ± 0.7 (15)

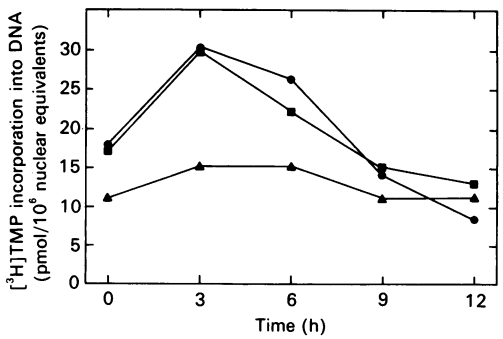

Fig. 1. Reversal of the DFMO-induced inhibition of DNA polymerase α activity by spermidine

HeLa cells were synchronized for S phase by the double-thymidine-block procedure in the absence or presence of 1 mM-DFMO. Spermidine (20 μM) was supplemented to one cell culture containing DFMO at 62 h of the synchronization procedure (10 h before initiation of S phase at 72 h). Nuclei were isolated from cells 3 h after the initiation of S phase and extracted with buffer A containing 0.3 M-KCl. Nuclear salt extracts of control (●), 'DFMO cells' (▲) and 'DFMO cells' supplemented with 20 μM-spermidine at 62 h (■) were assayed for DNA polymerase α activity at the indicated intervals after the initiation of S phase. DNAase I-'activated' calf thymus DNA was utilized as a template for the assay of DNA polymerase α. Each experimental point is the mean of assay results on triplicate samples, corrected for an unincubated blank. Variability of assay results between replicates was less than ±10%.

DNA synthesis by HeLa-cell nuclei reconstituted from subnuclear fractions

To attempt to characterize the deficiency in DNA synthesis by nuclei derived from HeLa cells depleted of polyamines, we have prepared subnuclear fractions by salt-extraction procedures introduced by Brun & Weissbach (1978) and extended by Enomoto *et al.* (1983*a*,*b*). The reconstitution of the nuclei, with respect to their capacity to synthesize DNA, was demonstrated by these investigators.

Optimum conditions for the salt extraction of HeLa-cell control nuclei were established in preliminary experiments (results not shown). The extraction of the nuclei at 4 °C for 30 min in buffer A containing 0.3 M-KCl was found to decrease DNA synthesis by the extracted nuclei substantially. The restoration of DNA synthesis by addition of nuclear salt extracts to salt-extracted nuclei was tested, and three nuclear equivalents of salt extract appeared to be optimal for reconstitution of the control nuclei. We selected this protocol for subsequent experiments. The necessity for the provision of excess salt extract for reconstitution may be related to enzyme instability or possibly incomplete uptake of DNA polymerase α in the salt extracts by the salt-extracted nuclei.

In contrast with the successful reconstitution of control HeLa-cell nuclei for DNA synthesis, salt extracts of nuclei from cells depleted of polyamines, or the nuclei from which these extracts were obtained, had only partial activity in supporting DNA synthesis in reconstitution experiments. The results of five nuclear reconstitution experiments, utilizing subnuclear fractions from control of polyamine-depleted cells, are summarized in Table 3. The decrease in DNA synthesis after the extraction of control or polyamine-depleted cell nuclei with 0.3 M-KCl was comparable, i.e. DNA synthesis by the extracted control nuclei was decreased by approx. 60%, and extracted polyamine-depleted nuclei synthesized 50% less DNA. The reconstitution of control salt-extracted nuclei with their salt extract averaged 77% of the activity for DNA synthesis of the intact nuclei. On the other hand, reconstitution of the control salt-extracted nuclei with the salt extract of polyamine-depleted cell nuclei ('DFMO nuclei') restored only 50% of the DNA synthesis assayed in the intact control nuclei. Thus the salt extract of the nuclei of polyamine-depleted cells contained lower activity of soluble DNA-replication enzymes than did salt extracts of the control nuclei.

Salt extracts of either control cell nuclei or 'DFMO nuclei' increased DNA synthesis by salt-extracted 'DFMO nuclei' only 11% and 5% respectively. Thus the chromatin of polyamine-depleted cells is not utilized as a template for DNA synthesis, regardless of the source of soluble DNA-replicating enzymes and factors (i.e. salt extracts of either control nuclei or 'DFMO nuclei') utilized in nuclear reconstitution.

DNA polymerase α activity in salt extracts of HeLa-cell nuclei

In HeLa cells, grown in complete medium in the absence of inhibitors, the activity of DNA polymerase α is highest during mid-S phase of the cell cycle (Chiu & Baril, 1975; Enomoto *et al.*, 1983*b*). The highest enzyme activity coincides with the peak of DNA synthesis observed at this time (Mueller & Kajiwara, 1969; Herbst & Elliott, 1981; Gallo *et al.*, 1986). We have utilized the assay of Enomoto *et al.* (1983*b*), optimized for DNA polymerase α, to determine the activity of this enzyme in salt extracts of HeLa-cell nuclei and lysates. Results shown in Fig. 1 demonstrate the profile of DNA polymerase α activity in HeLa-cell nuclei after the initiation of S phase. In control cell nuclei, the activity of DNA polymerase α reaches a peak at approx. 3 h after the initiation of S phase, whereas polyamine-depleted (by DFMO) cell nuclei do not show a similar elevation of the decreased 50% enzyme activity throughout the period of S phase. The addition of 20 μM-spermidine, to cultures synchronized in the presence of DFMO, 10 h before the initiation of S phase completely reverses the effect of polyamine depletion on the nuclear DNA polymerase α activity profile.

Characterization of deficiency in DNA polymerase α activity

Mammalian DNA polymerase α has been characterized as a 640 kDa holoenzyme complex (Vishwanatha *et al.*, 1986*a*). Modified DNA template preparations have been utilized to differentiate between the intact holoenzyme and enzyme complexes lacking one or two subunits (Lamothe *et al.*, 1981; Pritchard *et al.*, 1983; Vishwanatha *et al.*, 1986*a*,*b*). It was shown that DNA polymerase α holoenzyme utilizes DNA templates

Table 4. DNA polymerase α activities of nuclear salt extracts from control and DFMO-inhibited HeLa cells

HeLa cells were synchronized for S phase in the absence or presence of 1 mM-DFMO. Nuclei were isolated from cells 3 h after the initiation of S phase and extracted with buffer A containing 0.3 M-KCl. Salt extracts of nuclei were assayed for DNA polymerase α activity with either DNAase I-'activated' or heat-denatured calf thymus DNA as a template. The values are the means of assay results on triplicate samples of salt extracts of nuclei. Variability of assay results between individual samples was less than ±10%. Numbers in parentheses are the normalized results with activity of control nuclear salt extracts equal to 100%.

Source of salt extract	DNA template	DNA polymerase α activity (pmol of [^{3}H]dTMP incorporated/10^6 nuclear equivalents) Expt. 1	Expt. 2	Expt. 3
Control nuclei	'Activated'	23 (100)	13 (100)	17 (100)
'DFMO nuclei'	'Activated'	9.2 (40)	7.1 (55)	9.3 (55)
Control nuclei	Heat-denatured	23 (100)	10 (100)	13 (100)
'DFMO nuclei'	Heat-denatured	7.3 (32)	4.7 (47)	6.0 (46)

Table 5. DNA polymerase α activities of nuclear salt extracts and whole-cell lysates from control and polyamine-depleted HeLa cells

Salt extracts of nuclei and whole-cell lysates were prepared from HeLa cells synchronized in the absence or presence of 1 mM-DFMO 3 h after S phase was initiated. Salt extracts of nuclei and whole-cell lysates (nuclear salt extract and cytosol) were assayed for DNA polymerase α activity on DNAase I-'activated' calf thymus DNA as template. The values are the means of assay results on triplicate samples of respective extracts. Variability of assay results between individual samples was less than ±10%. Numbers in parentheses are the normalized results with activity of control salt extracts equal to 100%.

Source of salt extract	DNA polymerase α activity (pmol of [^{3}H]dTMP incorporated/10^6 nuclear or cellular equivalents) Expt. 1	Expt. 2	Expt. 3
Control nuclei	14 (100)	17 (100)	19 (100)
'DFMO nuclei'	9.7 (69)	6.6 (39)	8.5 (45)
Control lysate	19 (100)	16 (100)	14 (100)
'DFMO lysate'	17 (90)	17 (106)	14 (100)

containing high base/primer ratios (i.e. heat-denatured DNA), whereas an incomplete enzyme, lacking subunits such as C_1C_2 primer recognition proteins or DNA primase, has severely decreased activity on such templates. Both complete and modified subunit enzyme complexes synthesize DNA at similar rates on templates containing low base/primer ratios (i.e., DNAase I 'activated' DNA). The activity of DNA polymerase α in extracts obtained from nuclei isolated from control and polyamine-depleted HeLa cells was assayed by utilizing either heat-denatured or DNAase I-'activated' DNA templates. The results summarized in Table 4 indicate that DNA polymerase α activity in polyamine-depleted cell nuclei is not substantially decreased, relative to enzyme activity assayed on the 'activated' DNA template, when assayed on a heat-denatured DNA template. This suggests that the decreased enzyme activity in the polyamine-depleted nuclei is not caused by a modified subunit structure, but rather by a decreased nuclear content of the holoenzyme. Another possible explanation would be that post-translational modifications of DNA polymerase α, as described by Donaldson & Gerner (1987), have decreased holoenzyme activity in the nuclei of polyamine-depleted HeLa cells.

Several experiments were performed to compare DNA polymerase α activity in whole-cell lysates of control and polyamine-depleted HeLa cells with extracts of nuclei obtained from these cells. The activity of DNA polymerase α in control and polyamine-depleted whole-cell lysates is similar, whereas this enzyme activity of nuclei from polyamine-depleted cells is substantially lower than in control cell nuclei (Table 5). The results suggest that the uptake of DNA polymerase α by the nuclei of polyamine-depleted cells is decreased, or retention of the enzyme by these nuclei is diminished, as compared with control cell nuclei.

Attempted synchronization of HeLa cells by polyamine depletion

It has been established that the synthesis of mRNA coding for enzymes of polyamine biosynthesis, the modulation of ornithine decarboxylase activity and the concentration of cellular polyamines are under cell-cycle regulation (Heby *et al.*, 1976; Fuller *et al.*, 1977; Stimac & Morris, 1987). Seidenfeld *et al.* (1986) showed that DFMO interrupts cell-cycle traverse in human carcinoma cells, and accumulation of cells in G1 phase was demonstrated in four different cell lines. In our laboratory, Gallo *et al.* (1986) showed that polyamines must be added to HeLa-cell cultures at least 10–12 h before the initiation of S-phase DNA synthesis to reverse the interruption of cell-cycle traverse caused by polyamine depletion by DFMO. The addition of polyamines to these cells at later time points delayed the initiation of S phase, suggesting that polyamines are essential for progression through a specific control point of the cell cycle.

Thus it seemed likely that HeLa-cell cultures could be synchronized for S-phase DNA synthesis by utilizing a 'DFMO block' protocol. HeLa cells were grown in the presence of 1 mM-DFMO until proliferation ceased. At this time (96 h) cell viability in the culture was approx. 90% (Table 6). After 96 h, the culture medium containing DFMO was removed and replaced with fresh medium containing 20 μM-spermidine. Cell numbers were monitored to determine whether a synchronous peak of proliferation occurred. Results summarized in Fig. 2 demonstrate that cells in which proliferation was blocked by DFMO did not resume proliferation as a synchronized cell population when polyamine was replenished. These results suggest that the block of cell-cycle progression in polyamine-depleted cultures was not restricted to a narrowly defined control site. The asynchronous proliferation that occurred approx. 15 h after reversal of the DFMO block was indicative of a cell population, possibly arrested at diverse sites by a deficiency of polyamine cations, in a metabolic phase of the cell cycle encompassing most of G1.

Cell-cycle-specific nuclear protein phosphorylation: effect of polyamine depletion

It has been suggested that cell-cycle traverse through specific control sites, or commitment points, in the cell cycle of eukaryotic cells can be controlled by modulating protein kinase activity, possibly by phosphorylation (Murray, 1987; Lee & Nurse, 1987). In HeLa cells, variations were shown to exist in the phosphorylation of several non-histone proteins during cell-cycle progression (Song & Adolf, 1983). In order to provide some evidence that HeLa cells depleted of polyamines by DFMO might have an altered pattern of nuclear protein phosphorylation, we studied the phosphorylation of nuclear proteins *in vitro* in both control and polyamine-depleted HeLa cells before the initiation of S-phase DNA synthesis. Results illustrated in the

Table 6. Viability of HeLa cells in an unsynchronized cell culture

Unsynchronized HeLa cells were grown in the absence or presence of 1 mM-DFMO, and fresh medium was added every 24 h and cell density was adjusted to 300000 cells/ml. After 60 h, the cells grown in the presence of DFMO were divided into two separate cell cultures: spermidine (20 μM) was supplemented to one of the DFMO-containing cell cultures at this time. Cell viability was determined for each cell culture at the indicated time periods.

	Viability (%)			
Source of cells	24 h	48 h	72 h	96 h
Control	97	97	98	98
DFMO	97	97	96	90
DFMO + 20 μM-spermidine	–	–	96	96

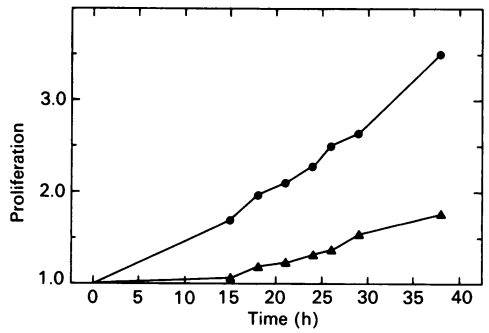

Fig. 2. Growth of DFMO-inhibited HeLa cells after removal of DFMO from the cell culture

Unsynchronized HeLa cells were grown in the absence or presence of 1 mM-DFMO until cell proliferation ceased in DFMO-containing cell cultures. At this time (0 h), cells were removed from medium containing DFMO and set at a density of 3×10^5 cells/ml in fresh DFMO-free medium supplemented with 20 μM-spermidine (▲). Control cells (●) were set at a density of 3×10^5 cells/ml in fresh medium. Cell proliferation was monitored at the time intervals indicated with a Royco automatic cell counter.

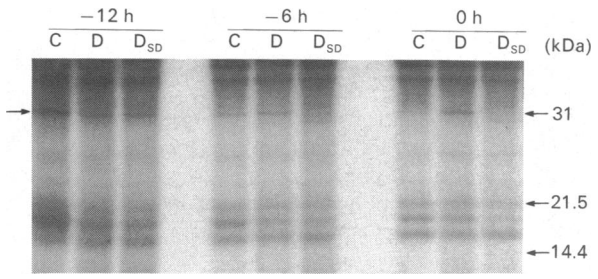

Fig. 3. Cell-cycle-specific nuclear protein phosphorylation: effect of polyamine depletion

Control (C), DFMO-supplemented (D) and DFMO-supplemented HeLa-cell cultures to which spermidine (20 μM) was added 12 h before the initiation of S phase (D_{SD}) were synchronized by the double-thymidine-block procedure. Nuclei were isolated and assayed for nuclear protein phosphorylation *in vitro* 12 h (lanes 1–3), 6 h (lanes 4–6) and 0 h (lanes 7–9) before S phase was initiated. The arrow indicates the 31 kDa nuclear protein.

autoradiogram of Fig. 3 indicate that the phosphorylation of a 31 kDa nuclear protein is variable during the 12 h before S-phase DNA synthesis in control and polyamine-depleted cells. At 12 h before S phase, the 31 kDa nuclear protein was phosphorylated to a similar degree in both control and polyamine-depleted cells. However, control cells exhibited progressively decreased phosphorylation of the 31 kDa protein at 6 h before and at the initiation of S phase, whereas polyamine-depleted cells continued to phosphorylate the protein. The addition of 20 μM-spermidine to the culture medium containing DFMO virtually eliminated the phosphorylation of the 31 kDa protein at S phase. The phosphorylation patterns of other proteins remained constant in both control and polyamine-depleted HeLa cells during this 12 h period. It is unclear whether the observed decrease in the phosphorylation of the 31 kDa protein as control cells approach S-phase is caused by decreased synthesis of the protein, decreased protein kinase or increased phosphatase activity, or a more rapid turnover of the phosphorylated 31 kDa protein. The results do suggest that HeLa cells depleted of polyamines cannot progress normally toward S-phase DNA synthesis, but rather become arrested during the 12 h time period before S phase. Since polyamines have been shown to modulate both specific protein kinase and phosphatase activities, and ultimately to affect the phosphorylation state of specific proteins (Ahmed *et al.*, 1983; Verma & Chen, 1986; Friedman, 1986), it is possible that altered phosphorylation of this 31 kDa nuclear protein, in response to the concentration of cellular polyamines, may be an essential regulatory event in cell-cycle traverse into S phase.

This is scientific contribution 1724 from the New Hampshire Agricultural Experimental Station.

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Received 15 April 1991/25 July 1991; accepted 6 August 1991