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Polyamines May Regulate S-Phase Progression But Not the Dynamic Changes of Chromatin During the Cell Cycle

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Abstract Several studies suggest that polyamines may stabilize chromatin and play a role in its structural alterations. In line with this idea, we found here by chromatin precipitation and micrococcal nuclease (MNase) digestion analyses, that spermidine and spermine stabilize or condense the nucleosomal organization of chromatin in vitro. We then investigated the possible physiological role of polyamines in the nucleosomal organization of chromatin during the cell cycle in Chinese hamster ovary (CHO) cells deficient in ornithine decarboxylase (ODC) activity. An extended polyamine deprivation (for 4 days) was found to arrest 70% of the *odc*$^-$ cells in S phase. MNase digestion analyses revealed that these cells have a highly loosened and destabilized nucleosomal organization. However, no marked difference in the chromatin structure was detected between the control and polyamine-depleted cells following the synchronization of the cells at the S-phase. We also show in synchronized cells that polyamine deprivation retards the traverse of the cells through the S phase already in the first cell cycle. Depletion of polyamines had no significant effect on the nucleosomal organization of chromatin in G_1–early S. The polyamine-deprived cells were also capable of condensing the nucleosomal organization of chromatin in the S/G_2 phase of the cell cycle. These data indicate that polyamines do not regulate the chromatin condensation state during the cell cycle, although they might have some stabilizing effect on the chromatin structure. Polyamines may, however, play an important role in the control of S-phase progression. J. Cell Biochem. 68:200–212, 1998.

Key words: polyamines; chromatin structure; micrococcal nuclease; cell cycle; apoptosis

Growing evidence indicates that the template activity of chromatin is reflected in its structure [Ege et al., 1975; Dupuy-Coin et al., 1976; Igo-Kemenes et al., 1982; Weintraub, 1985; Gross and Garrard, 1988]. In synchronized vertebrate cells, analyses of chromatin by digestions with micrococcal nuclease (MNase) or DNase I have revealed that the bulk chromatin [Prentice et al., 1985; Moreno et al., 1986; Laitinen et al., 1990], as well as the chromatin of specific genes [Moreno et al., 1986; Chen and Allfrey, 1987; Laitinen et al., 1990; Feng and Villeponteau, 1990; Laitinen and Hölttä, 1994], undergo structural modifications during the cell cycle. While the nucleosomal organization of specific genes (*hsp*, c-*fos*, c-*myc*, *odc*) has been found to undergo rapid structural changes upon transcriptional activation [Wu et al., 1979; Chen and Allfrey, 1987; Feng and Villeponteau, 1990; Laitinen and Hölttä, 1994], in bulk chromatin major changes are largely restricted to the DNA replicative phase [Riley and Weintraub, 1979; Annunziato and Seale, 1982; Moreno et al., 1986; Laitinen et al., 1990]. Several chromosomal proteins have been suggested to be responsible for these changes [Weintraub, 1985; Gross and Garrard, 1988; Adachi et al., 1991; Aubert et al., 1991; Wolffe, 1991]. However, one should not neglect the possible contribution of small diffusible factors [Eissenberg et al., 1985], like polyamines, which are known to bind strongly to DNA and affect its

Abbreviations: ODC, ornithine decarboxylase; MNase, micrococcal nuclease; PCC, premature chromosome condensation; HU, hydroxyurea.

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conformational state [Feuerstein et al., 1990; Xiao et al., 1991].

At the chromatin level, polyamines have been reported to promote the condensation of the 10-nm fibers to the 30-nm fibers in vitro [Makarov et al., 1987] and to enhance the stability of isolated core nucleosomes [Morgan et al., 1987]. Notably, Snyder [1989] also documented that the depletion of polyamines by inhibition of polyamine synthesis may alter the chromatin structure of mammalian cells in vivo. That polyamines may play a role in structural alterations of chromatin is further suggested by their ability to promote premature chromosome condensation (PCC) in mitotic-interphase fused cell systems [Rao and Johnson, 1971; Sunkara et al., 1983]. Furthermore, prolonged polyamine deprivation of polyamine-auxotrophic Chinese hamster ovary (CHO) cells [Pohjanpelto and Knuutila, 1982; Hölttä and Pohjanpelto, 1982] is known to cause major chromosomal aberrations, such as decondensation and fragmentation of mitotic chromosomes.

The key enzyme in the synthesis of polyamines is ornithine decarboxylase (ODC), which catalyzes the conversion of ornithine into putrescine [for reviews, see Tabor and Tabor, 1984; Pegg, 1986]. The activity of ODC shows two to three peaks during the cell cycle, i.e., in G_0/G_1, G_1/S and G_2/M phases [Heby et al., 1976; Sunkara et al., 1981; Laitinen et al., 1990]. The most marked increase in ODC and putrescine occurs prior to the initiation of DNA replication [Russel and Stambrook, 1975; Hölttä et al., 1979; Heby, 1981]. Thereafter, the higher polyamines, spermidine and spermine, synthesized from putrescine [Tabor and Tabor, 1984; Pegg, 1986] accumulate in S, G_2 and mitosis [Russel and Stambrook, 1975; Hölttä et al., 1979; Sunkara et al., 1981; Fredlund et al., 1995]. These changes in ODC and polyamines seem important for normal cell cycling, as the polyamine depletion of polyamine-auxotrophic, serum-free growing CHO mutants [Pohjanpelto, et al., 1981; Hölttä and Pohjanpelto, 1982; Anehus, et al., 1984; Pohjanpelto et al., 1994] and of cells exposed to inhibitors of polyamine synthesis results in cell arrest in either G_1 [Seidenfeld et al., 1986] or S/G_2 phases of the cell cycle [Anehus et al., 1984; Snyder et al., 1989].

In this study, we addressed the question of whether polyamines might be involved in the control of the nucleosomal organization of chromatin and cell cycle progression in *ornithine decarboxylase*-deficient (*odc*$^-$) CHO cells showing a normal response to serum growth factors [Hölttä et al., 1989]. The data show that depletion of polyamines causes only a slight destabilization of the chromatin structure, and that the chromatin of polyamine-depleted cells is still capable of undergoing cell cycle-dependent changes. The results further show that polyamines play an important role in promoting S-phase progression and may also facilitate the G_1-S transition.

EXPERIMENTAL PROCEDURES

Cells

The ornithine decarboxylase-deficient CHO cells [Hölttä et al., 1989], were cultured on plastic petri dishes (diameter 9 cm), in a 1:1 mixture of Eagle's medium (MEM) and F12 medium containing 10% fetal calf serum (FCS), 20 μM putrescine, supplemented with penicillin (125 U/ml) and streptomycin (125 μg/ml). The human erythroblastic K562 and mouse myeloma (NS-1/X63) cells were grown in RPMI medium with 10% FCS. The cells were checked for Mycoplasma using Hoechst fluorochrome 33258 (2.5 μg/ml), with negative results.

Cell Synchronization and Cell Cycle Analyses

For synchronization of *odc*$^-$ cells in G_0/G_1 subconfluent cultures were deprived of serum for 18–38 h [Laitinen et al., 1990]. The cells were then allowed to reenter the growth cycle by replating the cells (2.5×10^6 cells/dish) in the presence of 10% dialyzed fetal calf serum (dFCS) in MEM/F12 with or without putrescine.

For synchronization to S phase, exponentially growing cells were treated with hydroxyurea (2 mM) for 24 h [Moreno et al., 1986; D'Anna and Tobey, 1989]. Thereafter the cells were allowed to enter the cell cycle by the addition of fresh medium without hydroxyurea.

For analysis of the distribution of the cells in G_0/G_1, S and G_2/M phases of the cell cycle, their DNA was stained with ethidium bromide and analyzed by flow cytometry (FACScan; Becton-Dickinson, Mountain View, CA) using either the SFIT or SOBR model. The cell cycle progression after synchronization was followed by adding 8 μCi [^{3}H]-methylthymidine to the cultures (~40,000 cells/cm^2) at 2-h intervals and measuring the incorporation of radioactivity into DNA during a 30-min period [Laitinen et al., 1990].

Quantitation of Polyamines

To quantitate cellular polyamine/DNA ratio, cells were first disrupted by sonication in 2 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 5 mM dithiotreitol and 0.1 mM EDTA. Polyamines were analyzed from a cold $HClO_4$ extract of the homogenate by HPLC [Hyvönen et al., 1988]. DNA was determined from a hot $HClO_4$ extract as described by Giles and Myers (1965).

Isolation of Nuclei

In this study, 5×10^7–10^8 cells were collected by centrifugation at 400 *g* for 5 min and washed three times with phosphate-buffered saline (PBS). The nuclei were then isolated essentially as previously described [Moreno et al., 1986; Laitinen et al., 1990]. The cells were lysed in 1 ml of the lysis buffer, reticulocyte standard buffer (RSB; buffer A; 10 mM Tris–HCl; pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) supplemented with 0.5% Nonidet P-40 (NP-40), and the nuclei pelleted by centrifugation. The nuclei were washed with 1 ml of the lysis buffer, and three times with buffer A, and finally resuspended in 400 µl buffer A. Phenylmethylsulfonylfluoride (PMSF) (Sigma, St. Louis, MO) at a final concentration of 1 mM was included at all steps of nuclear isolation. All steps were carried out at temperatures between 0°C and +4°C.

Micrococcal Nuclease Digestion

The MNase (Boehringer Mannheim, Mannheim, Germany) digestion was carried out as described elsewhere [Moreno et al., 1986; Laitinen et al., 1990]. The isolated nuclei were resuspended to a density of $A_{\lambda = 260nm} = 10$–20, and the enzyme was added to a final concentration of 50–75 U/ml. After different times of incubation, an aliquot (100 µl) of the digestion mixture was removed and mixed with an equal volume of ice-cold 20 mM EDTA (pH 8.0) to stop the reaction. To check possible autodigestion, nuclei were resuspended in the reaction buffer and incubated without exogenous MNase at +37°C for 60 min.

Chromatin Precipitation by Polyamines

Chromatin was prepared from nuclei ($A_{\lambda = 260nm} = 10$; V = 0.1 ml) by gentle MNase digestion (7.5 U/l A_{260nm}) at +37°C for 30 min. Nuclear debris was removed by low speed centrifugation (400*g* for 1 min) [Weintraub, 1984]; 40 µl of the low-speed supernatant-containing chromatin was then incubated on ice for 30 min with different concentrations of putrescine, spermidine or spermine. Condensed chromatin was collected by centrifugation at 700*g* for 5 min; the pellets were dissolved in 500 µl 0.1 M NaOH. The samples were heated at +65°C for 15 min and the DNA content of dissolved chromatin was determined by A_{260nm} absorption.

Isolation of DNA

Organic DNA extractions were carried out as described earlier [Maniatis et al., 1984]. Alternatively, DNA was isolated by using protein salting-out method [Laitinen et al., 1994a]. After digestion with proteinase K, 5 M NaCl was added to a final concentration of 1.2 M and the tubes were vortexed for 30 sec. Thereafter, the precipitated proteins were removed by three successive centrifugations at 400*g*, 450*g*, and 500*g* for 15 min at +4°C. The resulting supernatant was transferred into new tubes, and DNA was precipitated by adding 2 vol of ice-cold ethanol.

End-Labeling of DNA

One microgram of high molecular mass DNA from CHO cells or 50 ng of marker DNA (λ DNA cut with *Hin*dIII and ΦK174 DNA cut with *Hae*III, respectively) (Pharmacia, Uppsala, Sweden) was incubated with 5 units of the Klenow-fragment of *Escherichia coli* DNA polymerase I at +37°C for 5 min. This was followed by an additional incubation at +37°C for 15 min in the presence of 2 mM of dATP, dGTP, and dTTP and 5 µCi of [^{32}P]-dCTP. Both incubations were carried out in 10 mM Tris–HCl (pH 7.5)–10 mM $MgCl_2$. The end-labeling reaction was stopped by the addition of 250 mM EDTA (pH 8.0) to a final concentration of 40 mM. Nonincorporated nucleotides were removed by gel filtration through Biogel P-30 column (BioRad, Hercules, CA).

DNA Electrophoresis

DNA fragments were analyzed on 1.6% agarose (5–10 µg DNA) or 8% polyacrylamide (0.5 µg DNA) gels. The nucleosomal organization of DNA from the digested nuclei was analyzed by electrophoresis on 1.6% agarose gels after staining with ethidium bromide (EtBr) and illumination with ultraviolet (UV) light. Fragment sizes were calculated using λ-phage and ΦK174 DNA digested with *Hin*dIII or *Hae*III (Pharmacia) as

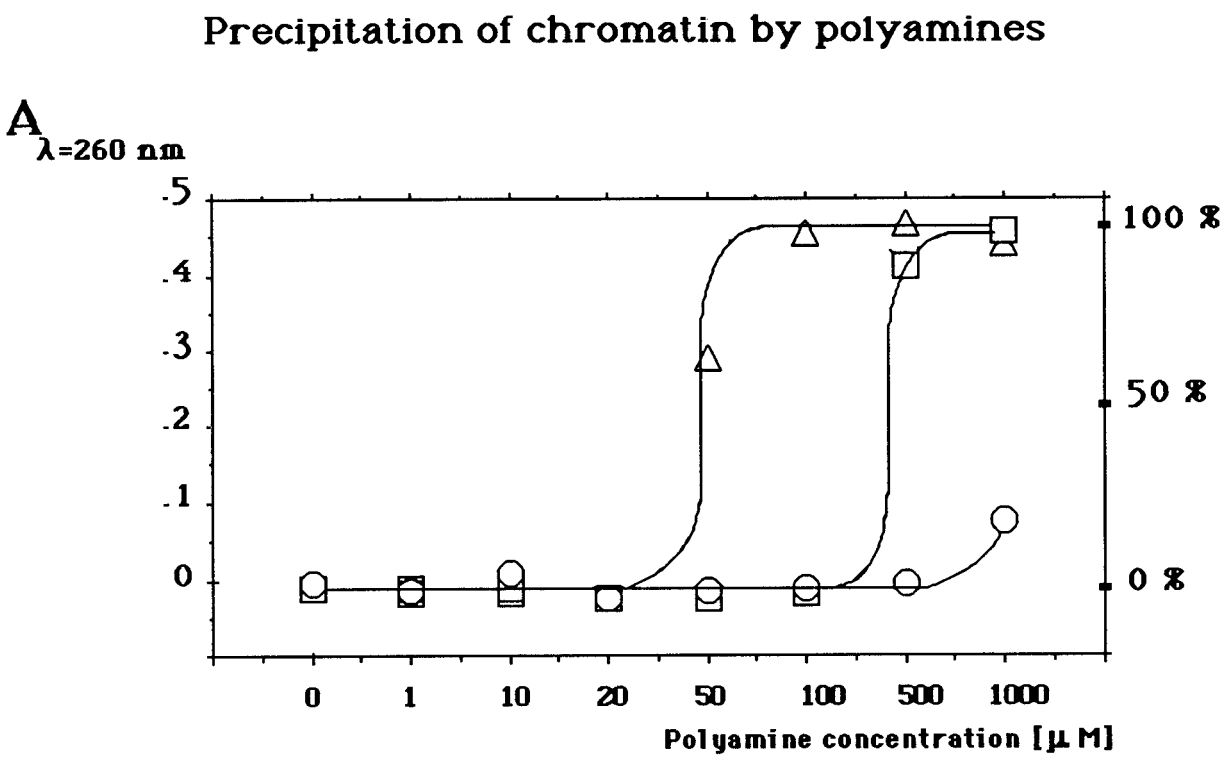

Fig. 1. Chromatin condensation induced by polyamines in vitro. Chromatin prepared from MNase-digested K562 nuclei was incubated with the indicated concentrations of putrescine (circles), spermidine (squares), or spermine (triangles), and the condensed precipitated chromatin was collected by centrifugation and dissolved in 0.1 M NaOH for determination of the optical density at 260 nm (left y-axis). Relative precipitation (%) indicated on the right y-axis. The x-axis indicates the polyamine concentration used.

a marker. The degree of chromatin digestion was analysed by densitometric scanning (EDC; Helena Laboratories, Beaumont, TX, or Hewlett-Packard, ScanJet Plus, Greeley, CO; Scan Analysis, Biosoft, Cambridge, UK).

RESULTS

Spermine Promotes Chromatin Condensation *In Vitro*

To study the possible functional role of polyamines in chromatin organization, we examined first the ability of different polyamines to cause chromatin condensation in vitro by monitoring the precipitability of the chromatin and its sensitivity to MNase digestion.

In the chromatin precipitation experiments, isolated chromatin (in average 5 kb in size) from the CHO or K562 cells was incubated in the presence of various concentrations of polyamines for 90 min at +4°C. Figure 1 depicts an analysis of the precipitation data and illustrates that 50% of the chromatin was precipitated with a 50-μM concentration of spermine. In the case of spermidine a much higher concentration (about 400 μM) was needed to achieve the same level of precipitation. In turn, putrescine appeared to be poor in precipitating the chromatin as it only caused a 20% precipitation of chromatin at a relatively high (1 mM) concentration (Fig. 1).

Next, we analyzed the effects of the exogenously added polyamines on chromatin condensation by MNase digestion analyses. Figure 2 shows that an increase in the polyamine content, especially in spermine (from 10 μM to 100 μM), rendered chromatin increasingly resistant to MNase. Spermidine proved to be less potent than spermine in promoting the condensation of the nucleosomal organization of chromatin (Fig. 2A,B), and putrescine (100 μM) did not protect the chromatin from the internucleosomal cleavage by MNase (Fig. 2B).

In Vivo Effect of Polyamine Depletion on the Nucleosomal Organization of ODC-Deficient CHO Cells

Even though polyamines appeared to exert profound effects on the chromatin structure in vitro, these results cannot be directly extrapolated to the situation in vivo. Therefore, we

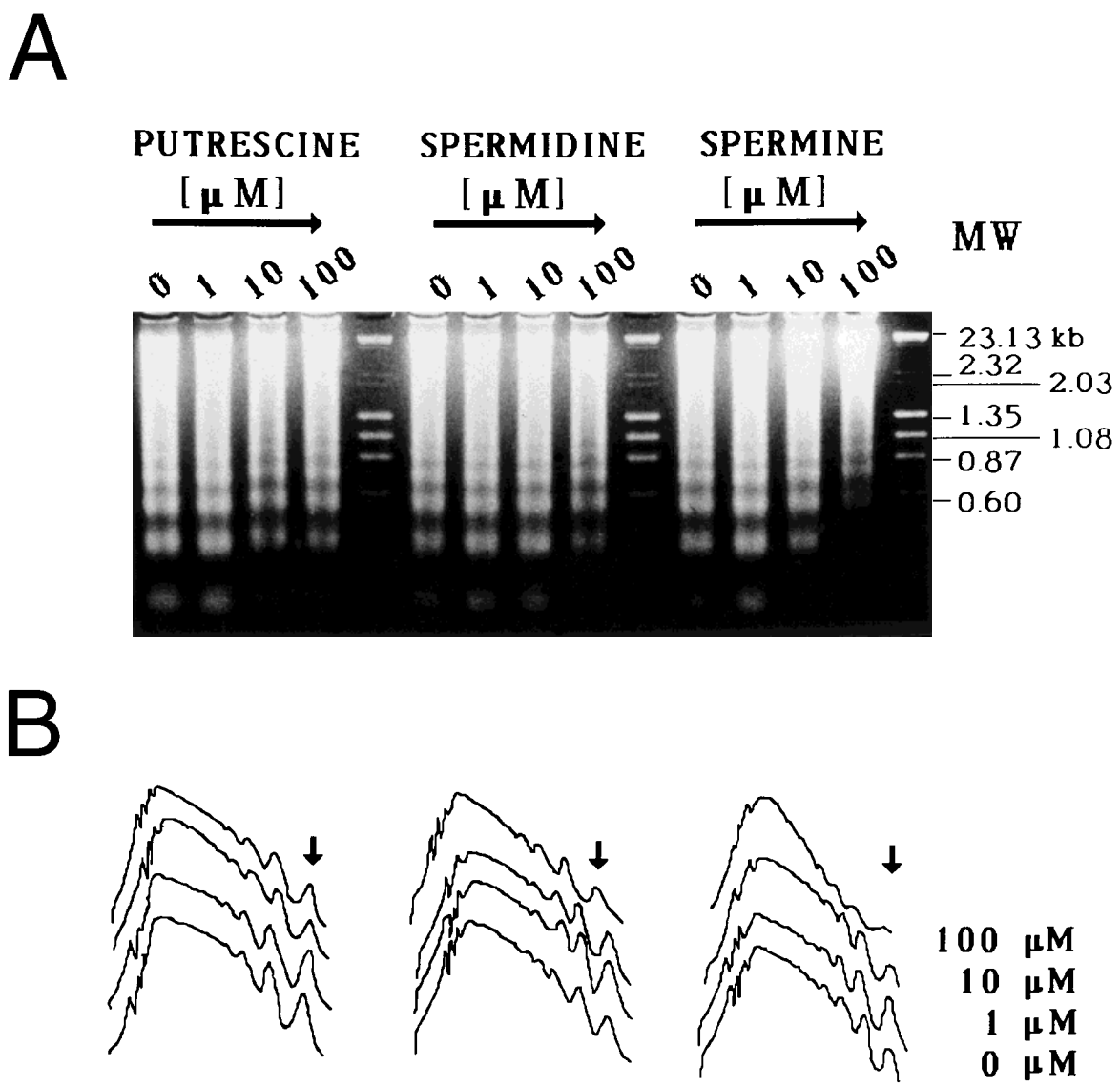

Fig. 2. Micrococcal nuclease digestion of nuclei incubated with polyamines. **A:** K562 nuclei were digested with MNase (75 U/ml; 30 min at +37°C) in the presence of various concentrations of polyamines. The DNA digests were then purified and electrophorezed on a 1.6% agarose gel and visualized by ethidium bromide (EtBr) staining and ultra violet illumination. The numbers on top indicate the concentrations of polyamines in the MNase digestions. MW indicates the sizes of molecular mass markers (λ DNA cut with *Hin*dIII and ΦK174 DNA cut with *Hae*III, respectively), loaded between the lanes of digested DNA (the unmarked lanes). **B:** The photographs (negatives) of the digestion patterns in A were scanned with a densitometer coupled to a computer. Migration is from left to right. The arrows point the position of the nucleosome monomer. Similar results were obtained in three independent experiments.

wanted to study whether the polyamine depletion is also associated with an altered nucleosomal organization of chromatin in vivo. For this study, we utilized an ODC-deficient CHO cell line unable to synthesize polyamines [Hölttä et al., 1989] to avoid the use of inhibitors and their possible nonspecific effects. These cells are routinely grown in a putrescine-containing medium, from which the cells can take up the polyamines needed for cell growth through an active transporter mechanism [Pegg, 1986]. Thus, to deplete the cells for polyamines one has to use polyamine-free medium and dialyzed serum (with polyamines removed). It is also notable that this cell line has maintained a normal regulatability by serum growth factors (see Fig. 6).

Figure 3 illustrates the changes in the polyamine levels in the mutant CHO cells grown for 1 or 4 days in the absence or presence of putrescine (20 μM). When the cells were deprived of putrescine for 1 day, the putrescine and spermi-

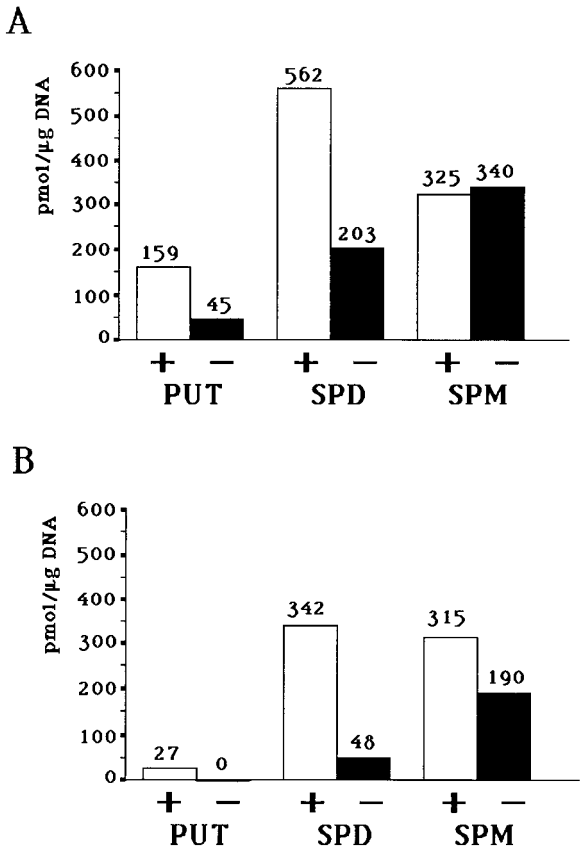

Fig. 3. Effect of putrescine-deprivation on the polyamine levels in ODC-deficient CHO cells. The cells were replated in fresh medium with 10% dialyzed fetal calf serum in the presence (+) or absence (−) of putrescine (20 µM) and cultured for 1 **(A)** and 4 days **(B).** Polyamine concentrations were determined from the perchloric acid extracts of cells as described under Materials and Methods. Values represent the mean of two parallel cultures. Similar results were obtained in two other independent experiments.

dine concentrations were decreased three- to fourfold, whereas the spermine levels remained unchanged. In cells grown for 4 days without putrescine the putrescine content was reduced to undetectable levels and spermidine and spermine concentrations were decreased 7- and 1.5-fold, respectively (Fig. 3). Coincidentally, the proliferation of polyamine-depleted cells was severely impaired, the cell number being only one-tenth of the control after 4 days of culture.

Next, we analyzed the effect of an extensive polyamine depletion on the cell cycle distribution and nucleosomal organization of chromatin in the ODC-deficient cells grown for 4 days in the absence or presence of putrescine. As shown in Figure 4, flow cytometric analyses revealed that the polyamine-depleted cells were largely arrested in the S phase; 70% of the cells were in the S phase, while the corresponding figure for the control cells was 30% (Fig. 4B). Nuclei were then isolated from the cells grown with or without putrescine and digested with MNase. Analysis of the DNA digests in agarose gels revealed that there was a marked difference in the chromatin structure between the polyamine-starved and -supplemented cells. Nuclei from the cells deprived of polyamines were much more sensitive to the MNase digestion than the control nuclei (Fig. 4C).

Having found that the polyamine-starved, asynchronously growing cells accumulated in S, we wanted to see whether the altered MNase sensitivity of chromatin was due to the polyamine depletion per se or to the cell cycle differences. To this end, the ODC-deficient CHO cells were grown for 4 and 6 days in the absence or presence of putrescine and were synchronized to the S-phase by treatment with 2 mM hydroxyurea (HU) for 24 h. Subsequent analyses of the chromatin structure of the MNase-digested nuclei indicated that deprivation of the cells of putrescine per se for 4 or 6 days did not significantly alter the chromatin structure (Fig. 5A,B). We also analyzed whether a long polyamine starvation might cause DNA fragmentation or apoptosis-like chromatin degradation in the cells. Nuclear DNA was isolated from the control and polyamine-starved cells (4 and 6 days), and the resulting DNA species were end-labeled with [^{32}P]-dCTP, and analyzed in 1.6% agarose gels. Putrescine depletion for 4–6 days did not result in a differential fragmentation of chromatin between the cells (Fig. 5C,D).

Polyamine Depletion Interferes With S-Phase Progression Already at the First Cell Cycle

The ODC-deficient CHO cells were synchronized by serum starvation for 24 h, and then stimulated to grow by the addition of 10% dialyzed FCS with one-half of the cells receiving 20 µM putrescine. The progress of the cells through the cell cycle was subsequently monitored by flow cytometry (Fig. 6) or by measuring the incorporation of ^{3}H-methylthymidine into DNA. Supplementation of putrescine appeared to slightly enhance the traverse of the cells from the G_1 phase to the S phase in three repeated, independent experiments (Fig. 6, and data not shown), but the significance of this small difference is difficult to say at present. The cells supplemented with putrescine also displayed a shorter S phase, and entered the second cell

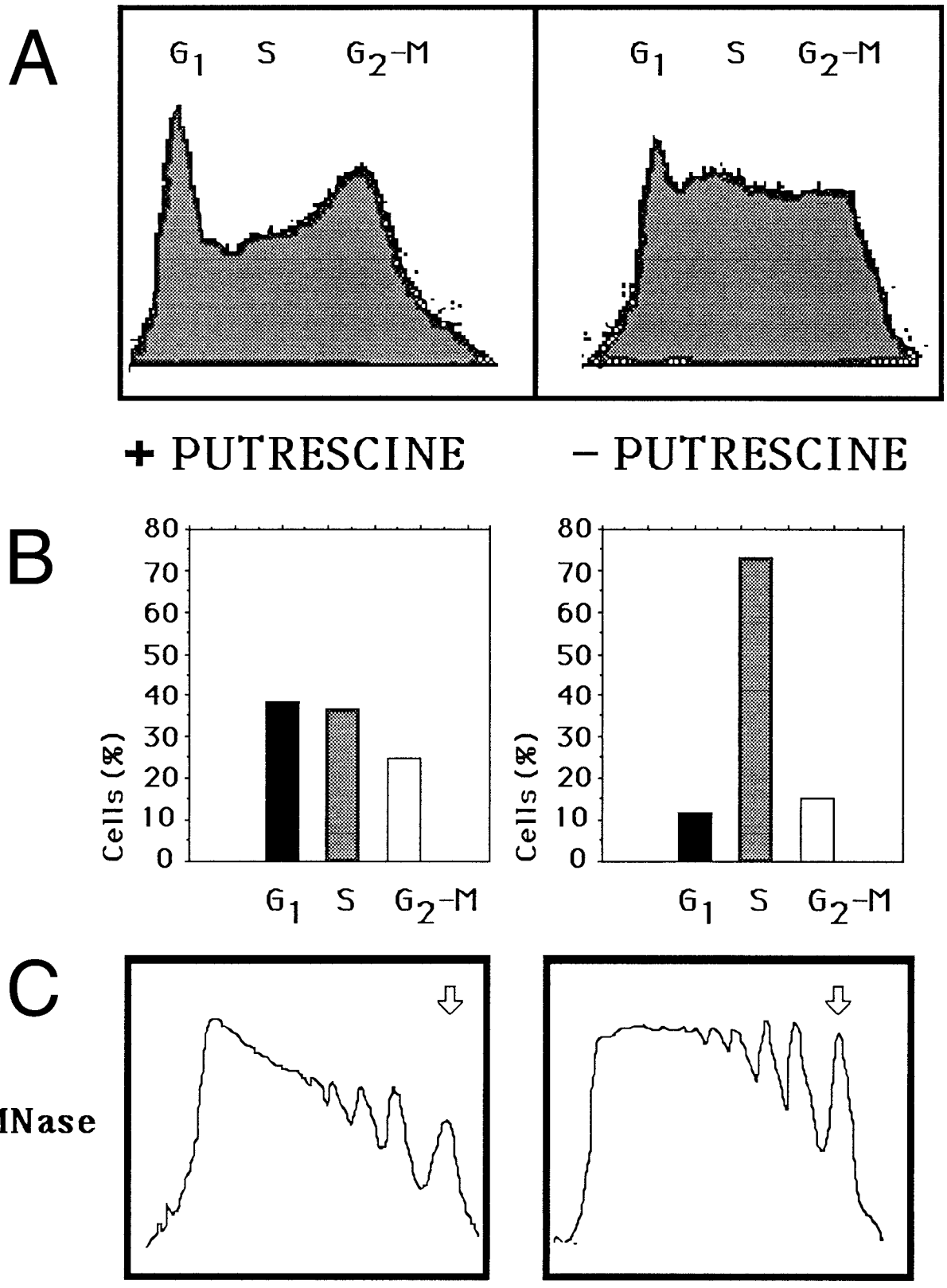

Fig. 4. Effect of polyamine starvation on the cell cycling and chromatin structure of ODC-deficient CHO cells. Asynchronous *odc*⁻ CHO cells were replated in fresh medium with 10% dialyzed fetal calf serum in the presence or absence of putrescine (20 μM). **A:** Flow cytometric profiles (FACS IV) of CHO cells grown for 4 days with (left) or without (right) putrescine. **B:** Cell cycle distribution analysis of the flow cytometric profiles in A. The SFIT model was used for the calculations. **C:** Nucleosomal organization of chromatin of the same cells. Nuclei from the cells grown with (left) or without (right) putrescine, were isolated, digested with MNase for 10 min, and the resulting DNA digests were analyzed on a 1.6% agarose gel. The DNA fragments were ethidium bromide-stained, ultraviolet-visualized, photographed, and scanned as in Figure 2. Arrows, positions of DNA from the nucleosome monomer. Migration is from left to right.

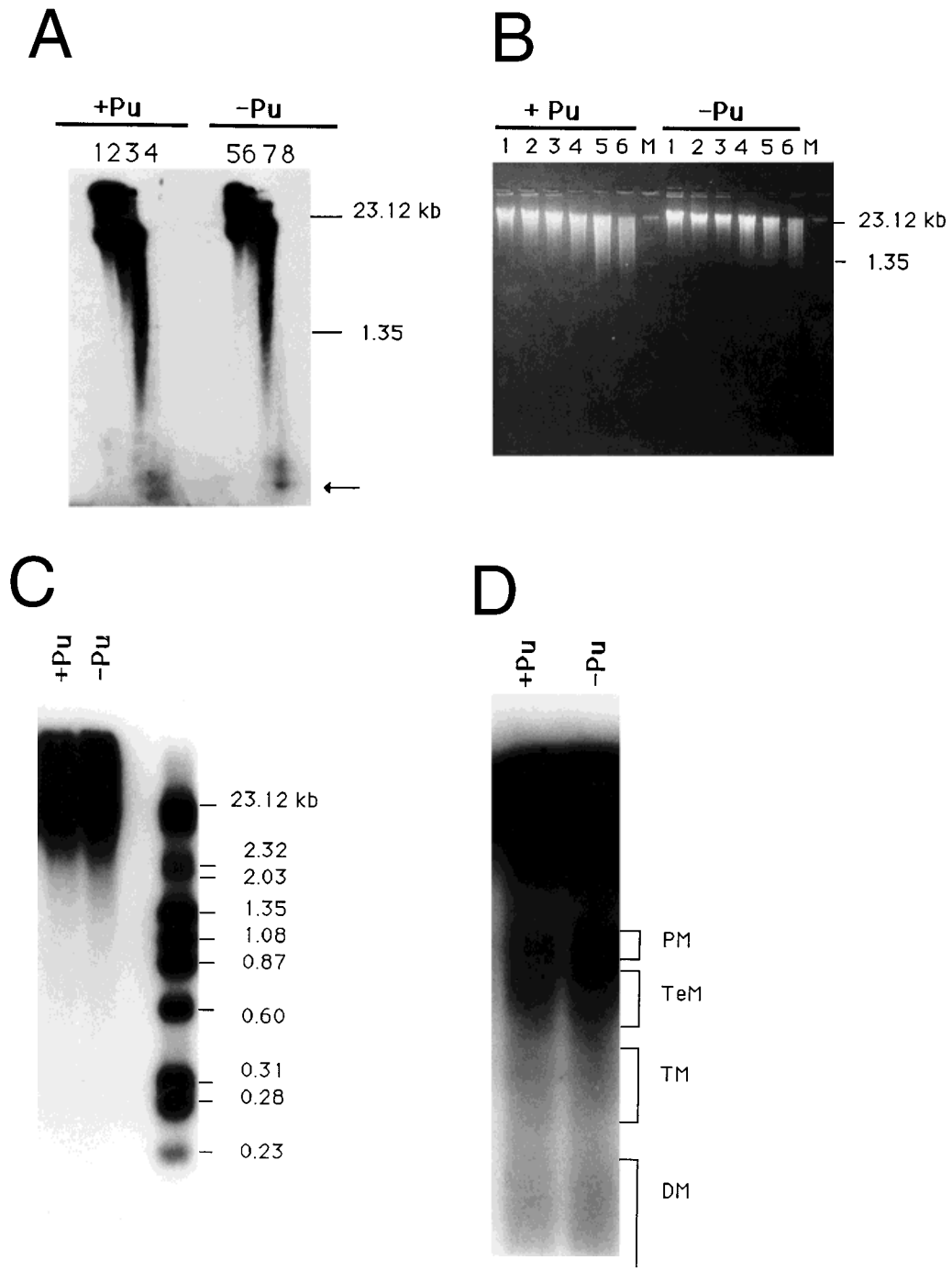

Fig. 5. Chromatin structure in S-phase-synchronized CHO-cells starved or not for polyamines. ODC-deficient CHO cells were grown in the presence (+Pu) or absence (−Pu) of putrescine for 4 or 6 days and then synchronized at S phase with 2.5 µg/ml hydroxyurea (2 mM) for 24 h. Nuclei from the cells were then isolated and subjected to micrococcal nuclease (MNase) digestion or autodigestion analyses. **A:** Nuclei from cells grown with or without putrescine for 4 days were digested with MNase (75 U/ml at +37°C), extracted for DNA, and 100 ng was applied on 1.6% agarose gels for electrophoresis. The DNA fragments were transfered to nylon filters and probed with total DNA labeled with [^{32}P]-dCTP. **Lanes 1 and 5,** undigested control DNA; **lanes 2 and 6,** 0.5 min; **lanes 3 and 7,** 1 min; **lanes 4 and 8,** 3 min; all represent digestion times with MNase. Right, sizes of molecular mass marker. Arrow, position of nucleosome monomer. **B:** Chromatin structure from CHO cells cultured with or without putrescine for 6 days. Nuclei were digested with MNase as above, the resulting DNA fragments electrophoresed on 1.6% agarose gels, stained with EtBr and illuminated by ultra violet. **Lanes 1 and 2,** undigested and autodigested (incubation without exogenous MNase at +37°C for 30 min) controls, respectively. Numbers 3 (2 min), 4 (3 min), 5 (5 min), and 6 (10 min) stand for various digestion times with MNase. Other symbols as in A. **C:** Direct end-labeling of bulk DNA from ODC-deficient CHO cells. Genomic DNA was isolated from the cells grown with or without putrescine for 4 days; 1.0 µg DNA was end-labeled and electrophoresed in a 1.6% agarose gel. ^{32}P-dCTP-labeled relative molecular mass markers (λ DNA cut with *Hin*dIII and ΦK174 DNA cut with *Hae*III) are indicated at the right. **D:** End-labeled bulk DNA from ODC-deficient CHO cells analyzed in a 8% polyacrylamide gel. The cells were grown in the presence or absence of putrescine for 6 days. DM, nucleosomal dimer; TM, trimer; TeM, tetramer; PM, pentamer.

cycle about three hours earlier than the putrescine-deprived cells (Fig. 6). When the progress of the cells through the cell cycle was monitored by measuring the incorporation of ^{3}H-methylthymidine into DNA, a similar pattern of changes in the cell cycle parameters was also seen (data not shown). Our data thus imply that polyamines enhance the traverse of the cells through the S phase and that prolonged polyamine starvation causes accumulation of the cells into the S phase (Figs. 4, 6).

Polyamine-Deprived Cells Are Still Capable of Condensing Chromatin at the S/G_2 Boundary

To see the possible effects of polyamines on the early dynamic changes of chromatin during the cell cycle [Laitinen et al., 1994], we isolated nuclei from the polyamine-supplemented and -starved cells at 0, 4, or 10 h of serum stimulation and subjected them to MNase digestion. These studies showed that polyamine depletion does not significantly interfere with the nucleosomal organization of chromatin during the G_1 and G_1/S phases of the first cell cycle (data not shown).

To evaluate to what extent polyamines might contribute to the chromatin condensation at the end of replicative phase, we assessed the ability of the polyamine-deprived cells to condense their chromatin during the late S phase when the chromatin of normal cells is known to undergo an extensive condensation [Moreno et al., 1986; Laitinen et al., 1990; Laitinen and Hölttä, 1994]. Cells were synchronized by deprivation of serum, and then stimulated to grow with serum in the absence or presence of polyamines. The progress through the cell cycle was monitored by measuring the incorporation of ^{3}H-methylthymidine into DNA. Nuclei were isolated at different times of the S-phase, digested with MNase and the DNA fragments analyzed on agarose gels.

As shown in Figure 7, involving an experiment different from that described in Figure 6, the nucleosomal organization of chromatin undergoes a marked condensation when the polyamine-deprived CHO cells traverse from the mid-S phase to the S/G_2 boundary of the first cell cycle. A similar pattern of changes in the chromatin structure to that in polyamine-depleted cells was seen in cells grown in the presence of polyamines (Fig. 7), but the time course of the events in S was accelerated (data not shown). These data indicate that factors other than polyamines are mainly responsible for the regulation of chromatin condensation.

DISCUSSION

Do Polyamines Affect the Nucleosomal Organization of Chromatin?

The data presented here show that polyamines affect the organization of chromatin in vitro. The chromatin precipitation analyses revealed that spermine was severalfold more effective than spermidine at condensing the chromatin and that putrescine had only a minor effect. These findings are basically similar to the other physicochemical analyses, like the linear dichroism, circular dichroism, and thermal denaturation studies, of the effects of polyamines on chromatin in vitro [Morgan et al., 1987; Makarov et al., 1987]. Similarly, our MNase digestion analyses demonstrate that polyamines stabilize or condense the nucleosomal organization of chromatin in vitro, with spermine being the most effective polyamine. These in vitro data indicate that polyamines (especially spermine) increase the stability of the nucleosomal organization of chromatin at linker DNA between the adjacent nucleosomes [Allan et al., 1980].

Several lines of evidence indicate that polyamines also play a role in chromatin condensation in vivo. Polyamine-depletion induced by a specific inhibitor, α-difluoromethylornithine (DFMO), is shown to inhibit premature chromosome condensation (PCC) [Sunkara et al., 1983], and difluoromethylornithine-treated CHO cells [Pohjanpelto and Knuutila, 1984; Pohjanpelto et al., 1988] or polyamine-auxotrophic CHO cells [Pohjanpelto and Knuutila, 1982] display highly decondensed or fragmented metaphase

Fig. 6. Flow cytometric analysis of the effect of polyamine deprivation on cell cycling. CHO cells deficient in ODC activity were synchronized by starvation for serum and putrescine for 24 h **(A)** or 38 h **(B),** and then triggered to grow by replating in fresh medium supplemented with 10% dialyzed fetal calf serum and 20 μM putrescine or without putrescine. **A:** Cell cycling profile of CHO cells grown in the presence of putrescine (20 μM; white areas) or without putrescine (gray areas). Numbers on right denote times after serum-stimulation. **B:** Profile of DNA synthesis in CHO cells as determined by measuring the percentage of cells in S-phase by flow cytometer using the SOBR model. Cells were grown in the presence (white circle) or in the absence (gray circle) of putrescine. For determining cells in S phase, at least 3,000 cells were counted, and the standard deviation (%SD) was less than 0.05%. A similar cell cycle result was obtained with ^{3}H-thymidine incorporation studies.

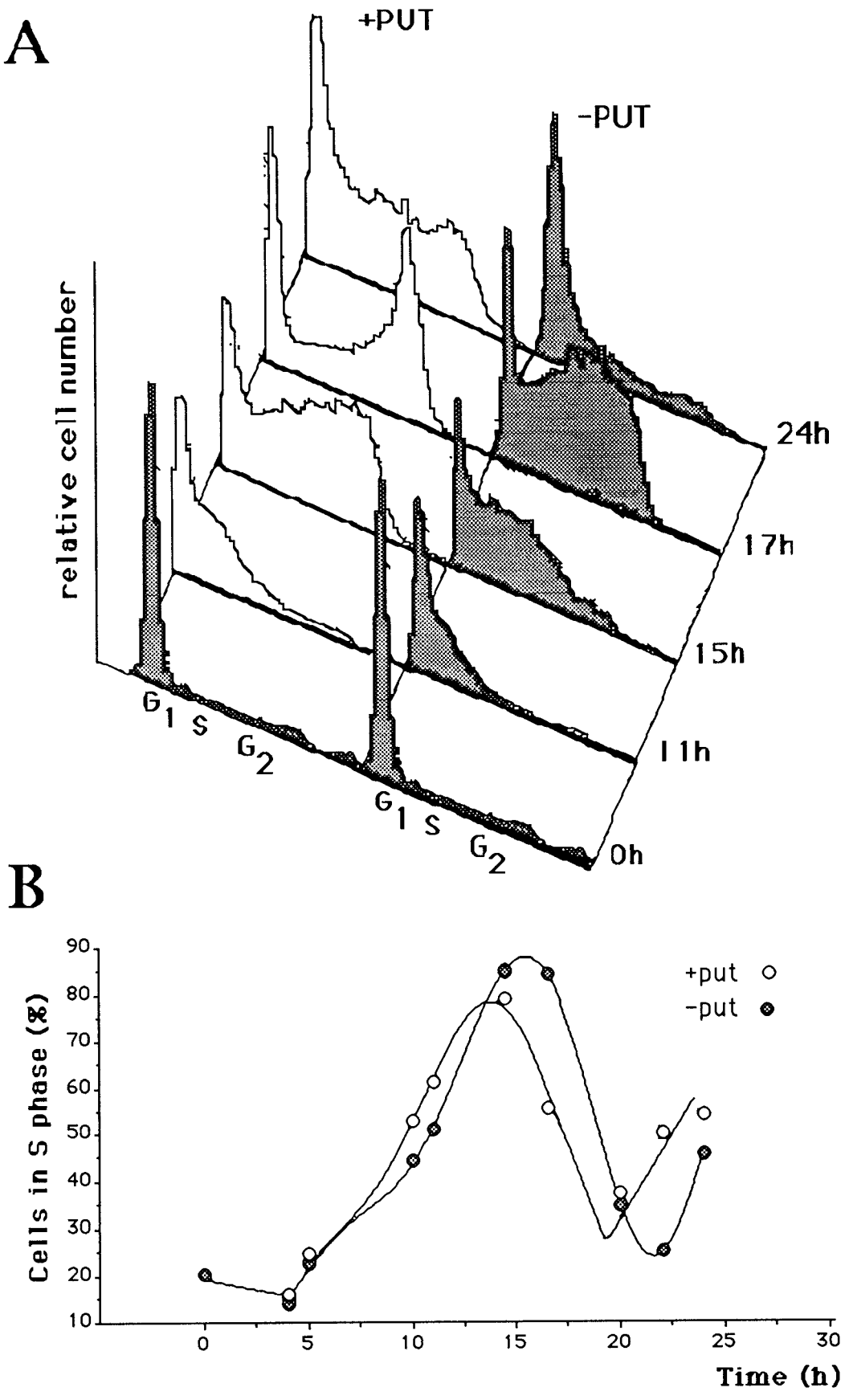
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Figure 6

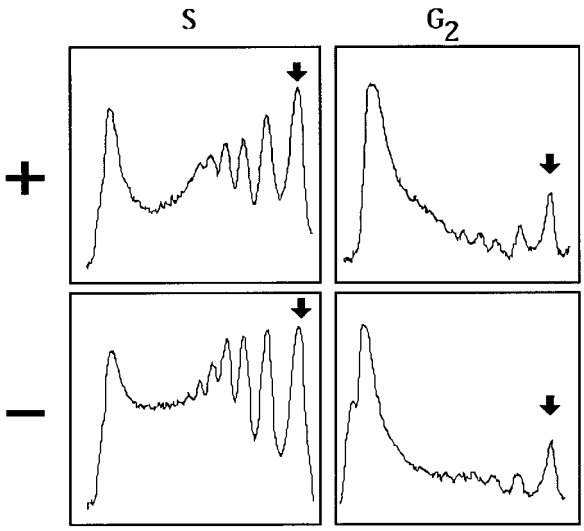

Fig. 7. Dynamics of nucleosomal organization changes in chromatin in polyamine-depleted cells during DNA replication. Cells were synchronized by deprivation of serum and polyamines for 38 h and stimulated to grow by adding 10% dialyzed fetal calf serum with (+) or without (−) polyamines. Nuclei were isolated from the synchronized cells at the S phase (S) and S/G_2-M phase of cell cycle (G_2) after serum stimulation, and digested with MNase for 20 min. The DNA fragments were analyzed by scanning as in Fig. 2B. Arrows, position of nucleosome monomer.

chromosomes after a prolonged deprivation of polyamines. Further, the difluoromethylornithine-treated HeLa cells have been reported to display an altered, nuclease-accessible chromatin structure [Snyder, 1989]. In line with these studies, we also found that a long polyamine depletion (4 days) of asynchronously growing ODC-deficient CHO cells was associated with an altered chromatin structure. The polyamine-deprived CHO cells displayed a markedly destabilized (MNase-sensitive) nucleosomal organization. In interpreting the data one should, however, take into account the possible cell cycle effects, as chromatin is known to undergo marked structural modifications during the cell cycle [Moreno et al., 1986; D'Anna and Tobey, 1989; Laitinen et al., 1990; Laitinen et al., 1994]. Indeed, our data show that the altered chromatin structure of polyamine-depleted cells can be largely attributed to the accumulation of cells in S-phase when the chromatin is not yet condensed.

Polyamine Depletion Interferes With S-Phase Progression

It has been reported previously that a prolonged polyamine starvation of polyamine-dependent, arginase-deficient [Anehus et al., 1984] and ODC-deficient [Pohjanpelto et al., 1994] CHO mutants that grow without serum, results in the accumulation of cells in S and G_2, and makes the cells incapable of going through mitosis. Here, we found that also the *odc*$^-$ CHO cells that respond normally to serum stimulation [Hölttä et al., 1989] are arrested in the S phase of the cell cycle after the polyamine depletion. In addition, we found that putrescine may play some role in G_1/S transition as supplementation of putrescine slightly accelerated the traverse of the cells through G_1 to the S phase. The latter finding is in good agreement with the report on mouse T lymphocytes, indicating that polyamines stimulate the transition of the G_0 lymphocytes to the S-phase [Kaminska et al., 1990].

Since polyamines appear to interfere with the cell cycle control mechanisms it is tempting to speculate that they might be involved in the regulation of the activity of cyclin-dependent protein kinases that are thought to be the master controllers of the G_1/S and mitotic events [Draetta and Beach, 1988; Fang and Newport, 1991; Lew et al., 1991]. This possibility is further supported by the existence of a correlation between the cell cycle-related fluctuations in the ODC activity [Heby et al., 1976; Sunkara et al., 1981; Laitinen et al., 1990] and the CDK2/CDC2 kinase activities [Draetta and Beach, 1988; Pines and Hunter, 1990; Lew et al., 1991].

In conclusion, our results are in agreement with earlier reports [Morgan et al., 1987; Makarov et al., 1987; Snyder, 1989] showing that depletion of cellular polyamines causes alterations in the structure of chromatin. However, our data show that these effects can be largely attributed to the arrest of cells in S-phase, and not directly to the changes in the polyamine content. It is also notable that polyamine depletion was found to markedly interfere with the cell proliferation before the observed alterations in chromatin structure took place. Furthermore, we found that in S–G_2 phase of the cell cycle, when the polyamine depletion had already perturbed the cell cycle, the chromatin of *odc*$^-$ cells could undergo a marked condensation. These data suggest that the alterations in polyamine levels do not play a critical role in the control of the dynamic changes of chromatin during the cell cycle. Thus, factors other than polyamines, possibly the chromosomal proteins such as topoisomerases [Adachi et al.,

1991] and proteins involved in transcription [Feng and Villeponteau, 1990; Lee and Garrard, 1991] and replication [Annunziato and Seale, 1982], seem to play the major role in the regulation of the chromatin changes during the cell cycle. However, the possible role of acetylation of polyamines, catalyzed by the same enzyme as the acetylation of histones [for references see Morgan et al., 1987], cannot be excluded by the present experiments. Finally, our data indicate an important role for polyamines in the control of cell cycle, specifically in promoting the S-phase progression, and elucidation of the underlying mechanisms remains an interesting object of further study.

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