Polyamine depletion is associated with altered chromatin structure in HeLa cells

Ronald D. SNYDER

Merrell Dow Research Institute, 2110 E. Galbraith Road, Cincinnati, OH 45215, U.S.A.

HeLa cells depleted of polyamines by treatment with α -difluoromethylornithine (DFMO), methylglyoxal bis(guanyldrazone) (MGBG) or a combination of the two, were examined for sensitivity to micrococcal nuclease, DNAase I and DNAase II. The degrees of chromatin accessibility to DNAase I and II appeared enhanced somewhat in all three treatment groups, and the released digestion products differed from those in non-depleted cells. DNA released from MGBG- and DFMO/MGBG-treated cells by DNAase II digestion was enriched 4–7-fold for Mg^{2+} -soluble species relative to controls. DNA released by micrococcal nuclease digestion from all three treatment groups was characterized as consisting of higher-order nucleosomal structure than was DNA released from untreated cells. At least some of the altered chromatin properties were abolished by a brief treatment of cells with polyamines, notably spermine. These studies provide the first demonstration *in vivo* of altered chromatin structure in cells treated with inhibitors of polyamine biosynthesis.

INTRODUCTION

Treatment of mammalian cells with inhibitors of polyamine biosynthesis leads to cessation of cell division. This anti-proliferative effect is apparently related to depletion of polyamines, since growth arrest is reversed by polyamine addition, but the exact role of polyamines in this process is still poorly understood, despite a tremendous amount of work in this area. In fact, although the polyamines putrescine, spermidine and spermine have been shown to affect the characteristics of a large variety of biochemical processes *in vitro* [1], no definitive role for polyamines *in vivo* has been established. Even though it is clear from studies *in vitro* that polyamines can substitute for inorganic cations in many reactions, our lack of knowledge regarding compartmentalization or intracellular localization of polyamines makes it very difficult to determine if these functions pertain to the situation *in vivo* as well. It is reasonable to assume that polyamines have cellular functions beyond those of simple cations, since it is well established that the biosynthesis and interconversion of these basic amines is highly regulated through several key enzymes, notably ornithine decarboxylase and S-adenosylmethionine decarboxylase (see [2] for review).

Among the more interesting characteristics of polyamines is their apparent interaction with DNA. Studies *in vitro* (reviewed in [3]) have demonstrated that polyamines, notably spermidine and spermine, can protect DNA from enzyme degradation and X-ray, thermal and alkaline denaturation, are involved in the conversion of B into A and Z forms of DNA, and aid in the condensation of DNA. The actual demonstration of an association of polyamines with mammalian cellular DNA, however, has been much more elusive, although spermine has been found to be associated with bacteriophage T4 DNA [4], and a nuclear localization of at least spermine is suggested in some studies with cultured

mammalian cells [5,6]. In addition, X-ray-diffraction studies coupled with computer-modelling analyses have revealed the possibility of specific polyamine–DNA interactions which could alter DNA structure in a fashion which would explain some of the known interactions of polyamines and DNA *in vitro* (see [7] for review). Other indirect evidence for a role of polyamines in chromatin structure and function is the finding of altered sensitivity of polyamine-depleted cells to various DNA-binding anti-tumour agents (reviewed in [8]).

Thus evidence exists suggesting that polyamines may play a role in chromatin structure. There is little evidence *in vivo*, however, in support of this theory. The purpose of the present study was to ascertain if modulation of cellular contents of polyamines could affect changes in chromatin structure in intact mammalian cells and to determine whether such changes could help to explain the manifold alterations in biochemical processes seen in polyamine-depleted cells.

MATERIALS AND METHODS

Cell culture and labelling

HeLa cells were seeded and maintained in Modified Eagle's Medium containing 10% (v/v) fetal-bovine serum or, in polyamine-supplementation experiments, 10% horse serum. Incubation was at 37 °C in a CO₂-controlled incubator. Cells were seeded at an initial density of 1.25×10^5 – 5.0×10^5 cells/60 mm-diam. tissue-culture dish. Unless specified otherwise, inhibitors of polyamine biosynthesis were added as follows: DFMO treatment was at 2.5 mM for 48 h; MGBG treatment was at 5 μ M for 24 h; DFMO/MGBG combined treatment was at the above concentrations, with MGBG added only during the last 24 h of DFMO treatment. Polyamine supplementation to cell cultures was for 3 h after the

removal of inhibitor, at a concentration of 50 μM (putrescine, spermine) or 10 μM (spermidine).

For some experiments, cellular DNA was prelabelled by a 48 h treatment of cells with [^3H]thymidine at 2.5 $\mu\text{Ci}/\text{dish}$.

Cloning efficiency studies

To determine the effects of inhibitor treatment on the ability of HeLa cells to form clones, appropriately treated cells were harvested with trypsin and seeded at a density of 250 cells/60 mm dish. Cells were allowed to grow for 10 days with a change of medium on day 5. They were then fixed with methanol, stained with Giemsa, dried, and the colonies counted. Untreated control cells exhibited 50–65% cloning ability by this protocol.

Polyamine determinations

Determination of cellular polyamines was accomplished by dansylation followed by reversed-phase h.p.l.c. as described in detail elsewhere [9]. A Waters system with two model 510 pumps, a model 680 automated gradient controller and a model 710B WISP Autoinjector was used.

Intracellular localization of polyamines

Compartmentalization of polyamines in HeLa cells was determined by a modification of the procedure of Mach *et al.* [6]. Briefly, cells were treated for 15 s with 1.5 ml of warmed buffer (pH 8.0) containing 0.4% Nonidet P-40. A portion of the lysate was made 10% with ice-cold trichloroacetic acid. A second 5 s wash of the nuclei still attached to the culture dish was treated similarly. Polyamines of the nuclei were extracted by a 1 h treatment with 10% trichloroacetic acid. All samples were extracted with trichloroacetic acid for 1 h, and clarified supernatants containing polyamines were then subjected to h.p.l.c. analysis for polyamines as described above.

Cell-cycle distribution

Determination of the proportion of the cell population in S-phase was accomplished by autoradiography. Appropriately treated cells growing on microscope well slides were pulsed for 1 h with 3 μCi of [^3H]thymidine (60 Ci/mmol) per 400 μl of Modified Eagle's Medium in each well. Slides were then processed for autoradiography by standard procedures. The percentage of S-phase nuclei was determined from counting no less than 500 nuclei from each Giemsa-stained slide.

Nucleoid sedimentation analysis

This was conducted by using generally accepted methodologies. Briefly, treated or control cells were harvested in phosphate-buffered saline and immediately added to a 300 μl lysis layer (containing 1.9 M-NaCl, 100 mM-EDTA, 32 mM-Tris/HCl, pH 8.0, and 0.5% Triton X-100) on top of a linear 15–30% neutral sucrose gradient. After incubation for 20 min gradients were spun for 45 min at 14000 rev./min in an SW 50.1 rotor at 18 °C. The position of the nucleoid band was determined under u.v. light. Variability was usually no greater than 10%. In experiments in which DNA supercoiling was estimated, the gradients also contained ethidium bromide at concentrations ranging from 1.5 to 14 $\mu\text{g}/\text{ml}$.

DNAase-sensitivity studies

Cellular DNA was prelabelled with [^3H]thymidine as described above. Nuclei were prepared by resuspension of harvested cells in buffer containing 10 mM-NaCl, 10 mM-Tris (pH 8.0), 2 mM-MgCl₂, 1 mM-dithiothreitol and 2% Triton X-100 for 15 min on ice. Cells were lysed by 12 strokes of a loose-fitting Dounce homogenizer. Nuclei were collected with three successive washes with buffer minus Triton X-100, and were estimated by light microscopy to be 90% free of cytoplasm.

For DNAase II digestion, nuclei were resuspended in 25 mM-sodium acetate at $1 \times 10^6/\text{ml}$, and 100 Kunitz units of enzyme was added for various periods of time at room temperature. The reaction was stopped by addition of 15 μl of 0.1 M-Tris, pH 11.8, and the reaction mixture was spun for 20 min in an Eppendorf centrifuge (6500 g). The nuclease-resistant DNA (pellet) was then either counted for radioactivity or stored for later use. The nuclease-sensitive DNA in the supernatant was either counted for radioactivity or, in some experiments, adjusted to 20 mM-MgCl₂ by slow addition of 1 M-MgCl₂. A 20 min Eppendorf spin separated the Mg²⁺-soluble 'transcriptionally active chromatin' in the supernatant from the Mg²⁺-insoluble 'heterochromatin' in the pellet.

For micrococcal nuclease (MNase) digestion, the nuclear isolation was as above except that the lysis buffer contained 5 mM-MgCl₂ and 3% Triton. Thrice-washed nuclei were resuspended in 10 mM-Tris buffer (pH 8.0) containing 10 μM -CaCl₂ at $2 \times 10^6/500 \mu\text{l}$, 0.03 Kunitz unit of MNase was added and the nuclei were incubated at room temperature. Samples were removed at various times, sedimented as above to separate nuclease-resistant and -sensitive DNA, and either counted for radioactivity or saved for sucrose-gradient analysis as described below.

Endogenous nuclease activity was monitored as follows: nuclei were prepared by lysis in buffer containing 250 mM-sucrose, 5 mM-CaCl₂ and 5 mM-Tris (pH 8.0), followed by homogenization as above. Nuclei were incubated at 37 °C for up to 24 h in physiological buffer containing 60 mM-KCl, 15 mM-NaCl, 1 mM-CaCl₂, 10 mM-MgCl₂, 1 mM-EDTA and 50 mM-Tris (pH 7.4). At various times, samples were removed, sedimented as above, and analysed by scintillation counting.

Conditions for all DNAase digestions were selected on the basis of published optimal reaction conditions, but with cognizance of the work of Walker & Sikorska [10] demonstrating the importance of maintaining chromatin in as near a native configuration as possible.

Nucleosomal-profile studies

The degree of digestion of DNA at the nucleosomal level was monitored after MNase treatment by sedimentation in neutral sucrose gradients. The post-Eppendorf supernatant (nuclease-sensitive DNA) was layered on top of a 12 ml linear 5–25% neutral sucrose gradient containing 20 mM-NaCl (adjusted to pH 7.2) and centrifuged for 18 h at 28000 rev./min in an SW 40 rotor at 4 °C. Fractions were collected by pumping from the bottom and were counted for radioactivity by liquid-scintillation spectrometry.

Nick-translation studies

Nuclei from non-radioactively labelled cells were isolated as described above and resuspended in buffer containing 50 mM-Tris (pH 8.0), 10 mM-MgCl₂, 100 mM-

dithiothreitol and 50 μg of bovine serum albumin/ml at $1 \times 10^6/400 \mu\text{l}$. Samples (100 μl) received 0, 0.05 or 0.5 μg of DNAase I and were incubated at 37 °C for 10 min and then chilled on ice. Nick translation was performed as described by Levitt *et al.* [11], with modifications. Nuclear pellets were resuspended in the above buffer (100 μl) containing 50 μM each of dATP, dCTP and dGTP, and 2 μCi of [^3H]TTP. Appropriate tubes received 3 Richardson units of *Escherichia coli* DNA polymerase I, and the reaction was allowed to proceed for 10 min at room temperature. The reaction was stopped by addition of 20 μl of 500 mM-EDTA, and the entire reaction mixture was applied to Whatman 3MM filters pre-wetted with 2% (w/v) $\text{Na}_4\text{P}_2\text{O}_7$. The discs were washed for 1½ h in five changes of 5% trichloroacetic acid, then washed with ethanol, and counted for radioactivity.

Chemicals

Putrescine, spermidine, spermine, MGBG, DNAase I type II, DNAase II type VII, MNase, dATP, dCTP and dGTP were obtained from Sigma Chemical Co. *E. coli* DNA polymerase I was from Boehringer-Mannheim. [$\text{methyl-}^3\text{H}$]TTP (60 Ci/mmol) and [$\text{methyl-}^3\text{H}$]thymidine (6.7 and 66 Ci/mmol) were obtained from ICN.

RESULTS

Treatment of mammalian cells with DFMO, MGBG or a combination of the two resulted in a significant alteration in cellular polyamine contents through interference with polyamine-biosynthesis pathways. Table 1 demonstrates the changes observed in HeLa cells after inhibitor treatment. Doses and treatment times used in these studies were those that produced optimal effects while having minimal cytotoxic activity. MGBG (5 μM) treatment for 24 h decreased cloning efficiency somewhat (38%), but this dose is below that reported to be associated with disruption of mitochondrial structure and function [12]. DFMO treatment depletes putrescine and spermidine to near zero, while leaving spermine contents largely unaffected. Upon removal of DFMO,

polyamine contents show no recovery for at least 3 h, but can be increased upon addition of exogenous polyamines. MGBG treatment depletes spermidine and spermine, but

Table 1. Effects of polyamine-biosynthesis inhibitors on polyamine contents of HeLa cells

Cellular polyamines were measured immediately after inhibitor treatment or after a 3 h incubation in the absence of inhibitor or in the presence of polyamines. Putrescine (PUT) and spermine (SP) were added at 50 μM ; spermidine (SD) was added at 10 μM . Normal polyamine contents in untreated HeLa cells were approx. 450 (PUT), 2400 (SD) and 3000 (SP) pmol/ 10^6 cells.

	Content (% of control)		
	PUT	SD	SP
Untreated	100	100	100
DFMO (2.5 mM, 48 h)	0.4	0.6	90
+ PUT 3 h	3300	37	74
+ SD 3 h	7	118	92
+ SP 3 h	8	6	169
+ all three	23	13	144
Minus DFMO 3 h	0.5	1	92
MGBG (5 μM , 24 h)	1405	56	37
+ PUT 3 h	2530	55	35
+ SD 3 h	820	78	29
+ SP 3 h	757	38	47
+ all three	1913	166	110
Minus MGBG 3 h	1400	58	39
DFMO/MGBG (48 h/24 h)	10	8	30
+ PUT 3 h	1870	14	53
+ SD 3 h	8	109	55
+ SP 3 h	23	10	62
+ all three	83	18	83
Minus DFMO/MGBG 3 h	14	4	22
Untreated (fresh media, 3 h)	300	125	98
+ PUT 3 h	384	98	93
+ SD 3 h	49	116	87
+ SP 3 h	64	98	109
+ all three	121	110	102

Table 2. Intracellular localization of polyamines in HeLa cells

Intracellular localization of polyamines was determined as detailed in the Materials and methods section. Data presented are the percentages of total cellular polyamines localized in the cytoplasm (Cyto) or nucleus (Nuc). Putrescine (PUT) and spermine (SP) additions were at 50 μM . Spermidine (SD) was added at 10 μM .

	PUT			SD			SP		
	% of total		Total (pmol/ 10^6 cells)	% of total		Total (pmol/ 10^6 cells)	% of total		Total (pmol/ 10^6 cells)
	Cyto	Nuc		Cyto	Nuc		Cyto	Nuc	
Untreated	100	0	155	83	5	1614	34	40	2700
+ PUT 3 h	92	0	4683	84	1	2148	48	24	1576
+ SD 3 h	83	9	120	83	4	2012	38	40	3432
+ SP 3 h	94	0	116	86	3	581	53	23	2920
DFMO (2.5 mM)	0	0	0	64	18	11	44	33	2455
+ PUT 3 h	95	1	773	90	1	1322	53	40	2520
MGBG (5 μM)	90	0	3548	79	5	1166	27	59	719
DFMO/MGBG	95	0	80	82	8	612	35	35	540

Fig. 1. DNA supercoiling in polyamine-depleted cells

HeLa cells treated with DFMO (2.5 mM, 48 h) or MGBG (5.0 μM , 24 h), or a combination of the two (D/M) were harvested and nucleoids were spun through neutral sucrose gradients containing increasing concentrations of ethidium bromide as described in the Materials and methods section. Spermine (SP) was added (panel c) at 50 μM for 3 h after removal of DFMO/MGBG. In all three panels, the data represent averages of two determinations for each ethidium bromide concentration.

markedly increases putrescine. This increase in putrescine has been previously examined and found to be due to the decreased conversion of putrescine into spermidine, owing to the MGBG-imposed block to the production of decarboxylated *S*-adenosylmethionine, and secondarily to the protection of putrescine from diamine oxidase, which activity is also inhibited by MGBG (see [13] for review). A combined treatment with the two inhibitors leads to depletion of all three polyamines, a condition which is again partially reversed with exogenous polyamine addition. The manner in which polyamines are depleted from inhibitor-treated cells and the uptake and interconversion characteristics under various conditions are consistent with previous studies (reviewed in [2]).

Owing to rapid redistribution upon cellular disruption, it is necessary to use very rapid cell-lysis techniques [6] to determine intracellular localization of polyamines. Table 2 shows that practically all cellular putrescine appears to reside in the cytoplasm even under conditions in which 40-fold normal contents exist. Spermidine, likewise, appears largely concentrated in the cytoplasm, although perhaps as much as 10% may be nuclear. On the other hand, as much as 30–50% of total cellular spermine appears to be nuclear. It has been previously shown that, under these isolation conditions, there is no preferential loss from nuclei of any of the polyamines during repeated washing [6]. Also, in studies not shown here, efflux of all three polyamines from nuclei which had been artificially loaded with polyamines appeared to follow similar

kinetics upon washing with buffer. These results argue against the possibility that different association constants with nucleic acids may contribute to the recovery of polyamines from the nucleus. Therefore, it appears that at least spermine is associated with the nucleus. This provides a rational basis for exploring the notion that polyamines interact with chromatin and exert effects similar to those seen in experiments *in vitro*.

Under the conditions employed in the present study, DFMO, MGBG and DFMO/MGBG treatments decreased the percentage of cells in S-phase as determined by autoradiography, by 26, 34 and 41% respectively (results not shown). A 3 h treatment with any of the three polyamines was not sufficient to alter significantly the fraction of S-phase cells. Flow-cytometric analysis did not indicate a significant increase in populations in G_0 – G_1 or G_2 – M phases. DFMO-treated cells exhibited no decreased ability to grow when plated at clonal density, whereas MGBG and DFMO/MGBG treatments decreased this capacity by 16 and 38% respectively. In these cases, however, a 24 h incubation period in the absence of inhibitor restored cloning efficiency to control values (results not shown). This indicates that, at these doses, inhibitor treatment is not overtly cytotoxic to HeLa cells. Thus any observed biochemical changes are not due to a toxic response.

The use of the nucleoid-sedimentation assay as it applies to the study of higher-order DNA structure (as described below) has been reviewed [14]. Basically, the degree of DNA supercoiling is determined from a series

of gradients containing increasing concentrations of the DNA-intercalating dye ethidium bromide. With increasing dye intercalation, nucleoids sediment more slowly as the DNA becomes more unwound (negatively supercoiled). Maximum unwinding is seen as minimal sedimentation. At higher dye concentrations, the DNA becomes positively supercoiled and begins to sediment faster as it takes on a more condensed form. Differences in the concentration of dye required to give minimal sedimentation between two DNA samples reflect altered supercoiling. Representative sedimentation profiles for untreated and inhibitor-treated cell cultures are shown in Fig. 1. In all three treatment groups, there is a slower rate of nucleoid sedimentation from treated cells over most of the range of ethidium bromide concentrations. Since there is no clear difference between control and treated cells in the concentration of ethidium bromide that produces minimal nucleoid sedimentation, it must be concluded that no alterations in supercoiling exist in polyamine-depleted cells. On the other hand, all treatment groups show slower sedimentation than controls at all ethidium bromide concentrations tested. In MGBG-treated cells, this same slower sedimentation is seen in the absence of ethidium bromide and could reflect DNA strand breakage.

Treatment of cells for 3 h with 1 mM-MgCl₂ failed to alter the sedimentation profile in intercalator-containing gradients, nor was it possible to reverse the pattern seen in DFMO/MGBG-treated cells by Mg²⁺ addition (results not shown). Thus, although Mg²⁺ is known to be involved in chromatin condensation, Mg²⁺ treatment had no effect on DNA-supercoiling profiles. In DFMO/MGBG-treated cells, however, a normal profile is restored by a 3 h treatment with spermine after inhibitor removal, strongly suggesting that spermine depletion is a causative factor in the altered chromatin structure seen in these experiments. Putrescine or spermidine treatment were ineffective in this regard (results not shown).

Radiolabelled nuclei were prepared from untreated or inhibitor-treated cells. In one series of studies, nuclei were incubated with DNAase II under conditions determined from the literature to be optimal, and at various times samples were removed for determination of nuclease-sensitivity. All treatment groups exhibited a time-dependent increase in DNA digestion. At digestion times of 30 min or longer, nuclei from all inhibitor-

treated groups showed greater digestion than did controls. Only in the case of MGBG, however, was this statistically significant. Little, if any, protection was afforded by a 3 h polyamine treatment in non-inhibitor-treated cells. However, as shown in Table 3, the increased DNAase II-sensitivity of MGBG-treated cells was completely abolished by a 3 h treatment with spermine. Putrescine and spermidine had no such effect.

DNAase II digestion of nuclear chromatin may also be used to distinguish template-active euchromatin from template-inactive heterochromatin, based on differential Mg²⁺-solubility of these two classes. Table 4 shows that the proportion of Mg²⁺-soluble DNA is very high relative to Mg²⁺-insoluble DNA in the DNAase II-sensitive fraction of MGBG- and DFMO/MGBG-treated cells. This is not seen in DFMO-treated or untreated controls. This ratio is elevated in DFMO/MGBG-treated cells regardless of the extent of DNA digestion, but in MGBG-treated cells it is more pronounced under conditions of limited digestion. Spermine addition to DFMO/MGBG- or MGBG-treated cells for 3 h before isolation of nuclei results in a Mg²⁺-soluble/-insoluble ratio more nearly like that seen in untreated cells (results not shown). Putrescine or spermidine addition did not have a consistent effect. Treatment with the polyamines themselves or with 1 mM-MgCl₂ (3 h) had no effect on the Mg²⁺-soluble/-insoluble ratio in otherwise untreated cells (results not shown).

MNase preferentially cleaves internucleosomal regions of chromatin generating, upon exhaustive digestion, mononucleosomes. Brief digestion, to less than 10% release of nuclear DNA, preferentially degrades template-active chromatin and generates a mixture of mono- and oligo-nucleosomal units. Unlike the case with DNAase II, the extent of MNase digestion was not found to differ significantly between inhibitor-treated and untreated nuclei (results not shown), but an analysis of the digestion products by sucrose-gradient sedimentation revealed interesting differences. Fig. 2 shows that, after all inhibitor treatments, a greater proportion of MNase-sensitive DNA was released in the form of oligonucleosomes (as opposed to mononucleosomes) than in the untreated control. In these studies, digestion was carried out for 2 min and resulted in 4% DNA digestion in all groups.

Table 3. Effect of polyamine addition on DNAase II sensitivity in MGBG-treated HeLa cells

DNAase II-dependent DNA digestion was measured either immediately after inhibitor treatment or after a further 3 h incubation in the presence of added polyamines (at usual concentrations). The data are averages $\pm$ S.E.M. of three determinations: *significant difference ($P = 0.05$).

Treatment	Digestion (%)		
	15 min	30 min	50 min
Untreated control	22 $\pm$ 4	42 $\pm$ 4	58 $\pm$ 5
MGBG (5 μ M, 24 h)	22 $\pm$ 2	57 $\pm$ 6*	72 $\pm$ 5*
+ Spermidine	23 $\pm$ 2	59 $\pm$ 3*	68 $\pm$ 2*
+ Spermine	22 $\pm$ 3	42 $\pm$ 3	56 $\pm$ 3
+ Putrescine	22 $\pm$ 1	62 $\pm$ 4*	68 $\pm$ 3*

Table 4. Mg²⁺-solubility of DNAase II-sensitive DNA from inhibitor-treated and untreated HeLa cells

Mg²⁺-soluble and -insoluble DNA released after DNAase II digestion was measured as described in the Materials and methods section. All inhibitor treatments were at the usual concentrations. Data from three experiments were pooled and grouped according to the absolute percentage DNA digestion (0–30%, 31–60%, 61–90%) to facilitate comparison of results. *Significant at $P = 0.001$ (compared with untreated control).

Treatment	Digestion ...	Mg ²⁺ -soluble/-insoluble ratio		
		0–30 %	31–60 %	61–90 %
Untreated		0.6 $\pm$ 0.1	0.7 $\pm$ 0.1	0.65 $\pm$ 0.3
DFMO		0.4	0.75 $\pm$ 0.3	0.8
MGBG		2.25 $\pm$ 0.6*	2.4 $\pm$ 0.3*	1.2 $\pm$ 0.3
DFMO/MGBG		3.75 $\pm$ 0.1*	4.2 $\pm$ 0.2*	3.4 $\pm$ 0.4*

Fig. 2. Nucleosomal profiles of MNase-digested control and inhibitor-treated HeLa nuclei

DFMO and MGBG treatments were performed as usual and the nuclei were isolated and briefly digested with MNase as described in the Materials and methods section. In all cases, digestion was carried out for 2 min and resulted in the release of 4% of total cellular DNA. The released DNA was analysed for its nucleosomal constitution by sucrose gradient sedimentation as described in the Materials and methods section. The arrow marks the location of mononucleosomes. Sedimentation was from left to right. Abbreviation: D/M, DFMO/MGBG double treatment.

With longer digestion times, DNA was digested primarily to mononucleosomes in all treatment groups (results not shown).

There was no difference observed between treated and untreated groups in the extent of digestion by endogenous DNAases over a 24 h period after removal of inhibitor (results not shown). This indicates that no detectable induction or enhancement of activity of cellular DNAase accompanies polyamine depletion. It further suggests that polyamine depletion does not lead to an increase in DNA strand breaks, which would serve as additional sites for exonuclease attack.

As a final probe of chromatin structure, nuclei were subjected to limited DNAase I digestion, followed by incubation in the presence of a DNA-synthesizing reaction mixture containing radiolabelled dTTP and *E. coli* DNA polymerase I. The *E. coli* enzyme initiates DNA synthesis at all DNA strand interruptions containing free 3'-hydroxy termini and extends this synthesis over relatively large stretches of template by a nick-translation process. Fig. 3 indicates that the incorporation of radiolabelled DNA precursor was higher in all inhibitor-treated groups than in untreated controls. There was no indication in any of the groups of incorporation in nuclei which had not received a prior DNAase treatment, again arguing against the possibility that inhibitor-treated cells had a higher initial content of DNA strand breaks, which served as template-primers for the *E. coli* polymerase.

Fig. 3. Nick-translation analysis of DNAase I-treated nuclei from control and inhibitor-treated HeLa cells

Isolated nuclei were treated with DNAase I at various concentrations, then permeabilized and subjected to nick translation by the *E. coli* DNA polymerase I as described in the Materials and methods section. Data shown are averages of two determinations, and are represented as the percentage increase in [^3H]TTP incorporated above that seen in nuclei of each group which did not receive the exogenous polymerase. In all groups, DNAase I treatment yielded a roughly 2-fold increase in incorporation even in the absence of added polymerase, presumably reflecting endogenous polymerase activity. In the absence of both DNAase I and exogenous polymerase, no radiolabel incorporation was observed. ○, untreated control; □, DFMO; ●, MGBG; ■, DFMO/MGBG.

DISCUSSION

That polyamines bind to chromatin in mammalian cells has been accepted largely on faith, as very little information exists regarding intracellular localization of polyamines. The logical extension of this paradigm envisages a role for polyamines in stabilization of chromatin structure, as an explanation for various phenomena seen in polyamine-depleted cells, such as inhibition of cell division [15], changes in differentiative state (reviewed in [16,17]), altered sensitivity to DNA-binding anti-tumour agents [7], and increased chromosome instability [18–20].

There has been only one published report directly demonstrating a possible chromatin alteration in polyamine-depleted mammalian cells. Hung *et al.* [21] showed that DNA isolated from DFMO-treated 9L rat brain tumour cells possessed a clearly increased visco-elastic retardation time compared with untreated cells, suggestive of a conformational change. This effect could be completely abolished by a further incubation of cells in putrescine-containing medium.

In the present study, an attempt was made to examine chromatin structure more extensively in polyamine-depleted HeLa cells. Two types of experiments were employed: (1) probing of DNA supercoiling by ethidium bromide intercalation, and (2) determination of sensitivity of chromatin to digestion by exogenous and endogenous DNAase.

Nucleoid-sedimentation analysis demonstrated no apparent alterations in DNA supercoiling in inhibitor-treated cells. DNA superhelicity therefore seems not to be affected by polyamine depletion.

Treatment with DNAase I (Fig. 3) and DNAase II leads to increased digestion of DNA from inhibitor-treated cells. The extent of this effect in all groups, though small, is similar to that reported for other systems in which experimental manipulations of cultured cells or animals have led to reported alterations in chromatin structure [22–24]. The increased digestibility of chromatin, as well as all other observed chromatin anomalies, are abolished by a 3 h incubation of cells with spermine, but not with putrescine or spermidine, suggesting a role of spermine depletion in altered chromatin structure. Since spermine is added only for the last 3 h, it is unlikely that this effect relates to competition for uptake of MGBG by spermine, but an alternative possibility may be that spermine displaces chromatin-bound MGBG, thus relieving some sort of MGBG-induced structural change. It may be important that alterations in DNAase II sensitivity are seen only at longer digestion times. This suggests that chromatin structure may not be affected in such a way as to produce a higher initial number of sites for DNAase II binding, but rather that digestion 'uncovers' additional sites, perhaps owing to relieving of conformational constraints.

Polyamine addition enhances the activity of at least some nucleases [25]. In our studies, polyamine addition to otherwise untreated HeLa cells did not result in increased chromatin digestibility, but cellular polyamines were not markedly increased under those conditions. It remains possible that the 40-fold increase in putrescine in MGBG-treated cells might have led to enhanced DNAase II activity, thereby causing MGBG-treated cells to show an apparently greater DNAase II accessibility than that seen in DFMO/MGBG-treated cells with maximally depleted polyamines. Thus digestibility is likely to depend on a number of variables, which are not easily distinguished in the type of experiments conducted here.

In addition to increasing nuclease sensitivity, polyamine depletion gives rise to a much more dramatic effect after DNAase II treatment. A marked increase in the proportion of Mg^{2+} -soluble relative to Mg^{2+} -insoluble DNA is associated with MGBG and DFMO/MGBG treatment, but not DFMO treatment alone (Table 4). It has also been possible to detect partial restitution (30–45%) of the normal Mg^{2+} -solubility profiles in DFMO/MGBG-treated nuclei which had been incubated in buffer containing spermine for 1 h before DNAase II digestion.

Altered Mg^{2+} solubility ratio may have profound biological consequences. Increased Mg^{2+} -solubility has been found to be associated with transcriptionally active chromatin [26,27] and the enrichment in that fraction of the high-mobility-group proteins, HMG14 and HMG17. It has been postulated [28] that these proteins may keep active chromatin in a soluble state under physiological conditions and thus increase its accessibility for transcription. Indeed, studies by Walker & Sikorska [10] have indicated that enhanced nuclease sensitivity of transcriptionally active chromatin may derive largely from its differential solubility, rather than some inherent structural change imposing increased sensitivity. Palvimo *et al.* [29] demonstrated that chromatin derived from polyamine-starved CHO-P22 polyamine auxotrophs (ODC⁻) contained nearly twice the amount of HMG14 as did controls, and that the degree of covalent modification (phosphorylation, ADP-ribosylation) of several HMG proteins was also different from normal

controls. It is tempting to speculate that the altered DNAase II-sensitivity observed in the present studies derives from an alteration in HMG proteins upon polyamine depletion. Perhaps loss of spermine from chromatin provides increased binding sites for HMG proteins. Subsequent addition of spermine might then displace the HMG protein and restore normal chromatin solubility in that area.

Digestion with MNase did not provide an indication of increased digestion in polyamine-depleted cells (results not shown), but DNA released after very brief digestions was of a clearly different conformation in treated and control groups. Whereas control DNA was enriched for mononucleosomes, DNAs from all treated groups displayed a marked tendency to accumulate as nucleosome oligomers (Fig. 2). Polyamine addition for 3 h before MNase digestion did not reverse these effects. It would appear, then, that perhaps altered MNase sensitivity is mediated by factors other than those involved in altered DNAase II sensitivity. It is of some considerable interest in this regard that cortical neuron cells from patients with Alzheimer's disease also show chromatin anomalies, among which is this same deficiency in the ability of MNase to digest chromatin to mononucleosomes [30,31]. These cells also display certain deficiencies in DNA repair, and DNA repair is also deficient in polyamine-depleted cells [32].

The above studies provide evidence that polyamines may play a role in chromatin structure *in vivo* which is consistent with their observed effects *in vitro*. However, the observed chromatin alterations could arise from factors other than polyamine depletion. Changes in cell cycling can be ruled out, since the depression of cell cycling after inhibitor treatment is not consistent with the enhanced sensitivity to nuclease digestion. Cytotoxic effects of MGBG through mitochondrial disruption would similarly not seem to be involved, since short spermine treatments readily reverse the effects. Finally, since inhibition of protein synthesis accompanies inhibitor treatment, it is possible that defective chromatin assembly could result from a deficiency of chromosomal proteins or modification of those proteins owing to a general depression of protein synthesis. Thus, although the present results are consistent with the notion that spermine depletion specifically results in altered chromatin structure, additional studies must be carried out to define more fully the nature and cause of the observed chromatin structural changes.

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