The effect of preincubation of HeLa cell nuclei with ATP on the degradation of mononucleosomal DNA by micrococcal nuclease

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ABSTRACT

HeLa cell nuclei with DNA labeled with [^3H] thymidine have been preincubated under varying conditions and then incubated with micrococcal nuclease. Aliquots, removed at increasing times, were analyzed for mononucleosomal size DNA and for acid-soluble DNA, the ratios were plotted and a slope was determined. Preincubation with ATP and a regenerating system increased the slope 2 fold. Optimum ATP concentrations were above 0.25 mM. The ATP effect was reversed by novobiocin. No inhibition of the ATP effect was observed with nalidixic acid, coumermycin, oxolinic acid, VM-26, aphidicolin, or 3 amino-benzamide. NAD or cAMP or cGMP had no effect with or without ATP. Other nucleoside triphosphates could substitute to varying degrees for ATP as could ATP analogues. Nuclei from log phase cells showed no ATP effect, but log phase cells, partially depleted of ATP by incubation with deoxyglucose, showed the effect. The effect was lost in nuclei on long-term storage. No evidence was found for differential degradation of core histones, histone H-1 or DNA, and there was no evidence of nucleosome sliding.

INTRODUCTION

The nucleosome, the basic unit of chromatin structure has been extensively characterized (see 1, 2 for review articles). The "core particle" consists of 145 base pairs of DNA wrapped in 1.75 superhelical turns around an octamer composed of two each of the 4 histones: H2A, H2B, H3 and H4. X-ray crystallographic maps of the core particles down to 7 Å resolution and of the histone octamer down to 3.3 Å resolution have recently been published (3,4). Histone H1 binds to the exterior of the particle, protecting an additional 10 base pairs of DNA at each end from nuclease digestion. This particle with H1, called the "chromatosome", contains 165 base pairs of DNA wrapped in two complete superhelical turns around the histone core. Chains of chromatosomes, separated by linker DNA of variable length, are coiled into a higher order structure called a solenoid. There are approximately 6 chromatosomes per turn of the solenoidal fibre which has a pitch of 11 nm and a diameter of 30 nm (2). Both electric dichroism and flow birefringence studies reveal edge stacked chromatosomes with faces inclined at an angle of between 20° and 32°

from the solenoid axis (5,6). The path of the linker DNA between chromatosomes is still entirely a matter of speculation (3,5,6,7,8,9). While details of the structure of chromatin have been elucidated, very little is known of the structural changes which must occur to permit cellular functions such as DNA replication, transcription, and repair. Because repair-incorporated nucleotides are initially nuclease sensitive, it was suggested that nucleosomes "unfold" during repair patch synthesis (10,11). "Unfolding" of isolated nucleosomes can be induced in vitro by intercalation of ethidium bromide (12), or by increasing (13) or decreasing (14) the salt concentration. As early as 1976, it was suggested that nucleosomes might unfold into two symmetrical halves (15), and recently such structures have been found in transcriptionally active regions (16) and in newly assembled minichromosomes (17). The association of nonhistone proteins with extended nucleosomes (16) makes it likely that different conformations of chromatin are mediated by cellular proteins.

Experiments in this laboratory had shown that in an in vitro system, the release of 3-methyladenine from the DNA of nucleosome core particles by purified glycosylases, was limited to approximately one third of the residues (18). Limitations in the release from linker regions of oligonucleosomes were also seen (19). Therefore our primary objective was to develop an assay which could be used to screen extracts for nucleosome "unfolding" activity, based on the assumption that on unfolded nucleosomes, the DNA would be more easily degraded by micrococcal nuclease. A number of groups had reported that ATP was required to get efficient DNA repair synthesis (20-22). In the event that ATP might stimulate nucleosome unfolding, nuclei were preincubated with ATP and an ATP regenerating system. Contrary to expectations, ATP was found to stabilize nucleosome structure when the recovery of monomer DNA was examined during the course of a digestion by micrococcal nuclease. At approximately the same level of acid soluble material, twice as much DNA was found in mononucleosome size fragments in nuclei preincubated with ATP at 37°C as in the nuclei preincubated at 37°C without ATP.

MATERIALS AND METHODS

Cell growth

HeLa cells were grown in Joklik's MEM supplemented with 6% horse serum. Cells were labelled for several generations with 0.1 mCi to 0.3 mCi [³H]thymidine per litre. Cells were collected 2 to 5 days after adding the [³H]thymidine, washed once with PBS, and stored at -20°C until needed. Cells held in stationary phase after initial labeling gave the best effect with ATP as discussed below.

Preparation of nuclei

Hela cell nuclei were prepared essentially as described by Butt *et al.* (23). PMSF was added to all buffers immediately before use to a final concentration of 0.5 mM. All operations were performed at 0-4°C. The thawed cells were resuspended with 0.32 M sucrose, 2 mM $MgCl_2$, 1 mM KPO_4 , pH 6.8, at about 10^7 cells/ml, and collected by centrifugation at 1000 x g for 6 minutes. The pellet was resuspended at 10^7 cells/ml with 10 mM NaCl, 1 mM KPO_4 , pH 6.8, and left on ice for 15 minutes. The swollen cells were harvested by centrifugation at 800 x g for 10 minutes, and resuspended with 0.35 M sucrose, 1 mM $MgCl_2$, 1 mM potassium phosphate, pH 6.4. The suspension was homogenized with 12 strokes of a loose fitting teflon pestle, and the nuclei were pelleted by centrifugation for 10 minutes at 800 x g. The nuclei were homogenized again as above, and then washed twice with 0.2 M sucrose, 80 mM NaCl, 5 mM Tris, pH 7.5, and collected by centrifugation at 1500 x g for 15 minutes. The nuclei were resuspended at about 3 mg/ml in the above buffer, to which EDTA and glycerol were added to final concentrations of 5 mM and 50% respectively. The nuclei were stored at -20°C, and used within a week after being prepared because the ATP effect was occasionally labile on longer storage.

Preincubation and nuclease digestion of nuclei

The nuclei were collected by centrifugation at 3500 x g for 15 minutes, and washed three times with 0.2 M sucrose, 80 mM NaCl, 0.5 mM PMSF, 5 mM Tris, pH 7.5, to remove EDTA and glycerol. The nuclei were then resuspended in 90 mM Hepes, 12 mM $MgCl_2$, 48 mM KCl, 0.5 mM PMSF, pH 7.4 (Buffer 1). Concentration was determined from optical density at 260 nm (20 units = 1 mg DNA). A typical incubation mixture of 1 ml contained 0.25 volume of Buffer 1 with nuclei, and had final concentrations as follows: DNA at 1 mg/ml, 40 mM Hepes, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, pH 7.6. ATP and PEP at 2.5 mM and 10 mM respectively, and pyruvate kinase at 4 units/ml were added to the nuclei where indicated. After 30 minutes at 37°C, $CaCl_2$ and micrococcal nuclease were added to final concentrations of 10 mM and 2 µg/ml. Two 50 µl aliquots were removed at the same time at one to two minute intervals and added to either 50 µl 20% TCA or 10 µl 0.1 M EDTA pH 8. After 30 minutes on ice, the TCA containing samples were centrifuged for 15 minutes in an Eppendorf microfuge. The supernatant fractions were removed and the radioactivity was determined by liquid scintillation counting. The pellets received 100 µl of 10% TCA and were heated at 100°C for 15 minutes then transferred to scintillation vials and counted. The percent acid soluble counts were calculated as the percent of the total counts recovered. The EDTA containing samples

were treated at 37°C with 0.5 mg/ml ribonuclease A for 30 minutes, followed by an additional 30 minutes with 1 mg/ml proteinase K and 0.4% SDS. The DNA was precipitated by adding 0.1 volume 3 M sodium acetate pH 5.5 and 2 volumes ethanol. After 30 minutes on dry ice, the DNA was collected by centrifugation in an Eppendorf microfuge at 4°C. The pellets were air-dried, then resuspended with 30 μ l 5 mM Tris, 2.5 mM EDTA, pH 8, 5 M urea, 1% SDS, 0.5% TBE buffer (90 mM Tris, 90 mM boric acid, 3 mM EDTA, pH 8.3) 0.01% bromophenol blue. The DNA was denatured by heating at 100°C for 3 minutes so that single strand cuts in the mononucleosome size DNA would not be measured. The samples were loaded on 8% acrylamide: bisacrylamide (29:1) gels containing 7 M urea and 0.02% SDS and TBE buffer. Running buffer was TBE containing 0.1% SDS. The gels were electrophoresed at 250 volts for 2 $\frac{1}{2}$ hours until the bromophenol blue running dye was 1 to 2 cm from the bottom. The gels were stained with toluidine blue (80 mg/l) dissolved in 40% methanol (v/v). Nucleosome size DNA, prepared from chicken erythrocytes as described by Price *et al.* (22), was run as a molecular weight standard. The bands corresponding to mononucleosome size DNA were cut out of the gel. The bands were wide enough to include DNA of 140 to 180 nucleotide residues. The piece of acrylamide (5 mm wide x 15 mm long x 1.5 mm thick) was finely minced and transferred to a glass scintillation vial. After adding 100 μ l of 3 M NaOH and 2.5 ml 50% H₂O₂, the vial was tightly capped and incubated at 68°C for 18 to 24 hours to dissolve the gel pieces. The sample was neutralized by adding 3 M HCl followed by 10 ml scintillation fluid. The radioactivity was determined in an LKB scintillation counter. The counts in the TCA pellets and corresponding supernatant fractions were added, and the mean total count in a 50 μ l aliquot determined. The percent acid soluble DNA and the percent mononucleosome size DNA at early time points were calculated and plotted relative to each other. ATP levels of perchloric acid extracts were measured either with the usual hexokinase, glucose-6-phosphate dehydrogenase system (24), or by Dr. S. Berger using a more sensitive amplification system (25).

RESULTS

ATP effect

The purpose of these experiments was to develop an assay that could be used to detect conformational changes in nucleosomes, more specifically, the "unfolding" which must occur to allow the enzymes involved in repair, replication, and transcription access to the DNA. The assay was based on the assumption that if nucleosomes "unfold", the DNA on the core particle would become

Figure 1. Results of a typical micrococcal nuclease digestion of nuclei after a 30 minute preincubation at 37°C without ATP. (a) Percent acid soluble DNA plotted versus time. (b) Percent mononucleosome size DNA plotted versus time. (c) Percent mononucleosome size DNA plotted versus percent acid soluble DNA. The data obtained at 15 and 20 minutes was not included in the determination of the slope by the least squares method.

sensitive to micrococcal nuclease. If that is the case, two predictions can be made: (1) with "unfolded" nucleosomes, there should be an increase in DNA degradation, measured simply as an increase in acid soluble material, and (2) with "unfolded" particles, there should be a decrease in the amount of monomer size DNA recovered by gel electrophoresis.

By using nuclei whose DNA has been uniformly labelled for several generations with [^{3} H]thymidine, it was possible to measure the release of acid soluble material at several points during the course of a digestion with micrococcal nuclease. In a typical experiment, the percent of acid soluble DNA increased throughout the 20 minute nuclease digestion (Figure 1a). The percent of DNA recovered in single strand mononucleosome size fragments at the same time points increased for the first 12 minutes when micrococcal nuclease digested primarily the linker regions between particles (Figure 1b). By 20 minutes, the level of mononucleosome size DNA was decreasing as micrococcal nuclease digested the DNA on the nucleosome core (Figure 1b). When the percent of mononucleosome size DNA is plotted versus the percent of acid soluble DNA, the points were approximately linear for the early part of a digestion with micrococcal nuclease (Figure 1c). If the nucleosomes unfolded, the slope of this line would be expected to decrease, since there would be a simultaneous increase in the percent of acid soluble DNA and decrease in the percent of mononucleosome size DNA.

Nuclei, isolated as described in Materials and Methods, were incubated at

Figure 2. Profiles of single stranded DNA isolated from nuclei preincubated with or without 2.5 mM ATP and 10 mM PEP and then incubated with micrococcal nuclease until 10 percent of the DNA was acid soluble. The preincubation was at 37°C for 30 minutes. DNA was isolated as described in the text and electrophoresed in 7 M urea in an 8% acrylamide gel. The direction of electrophoresis was from right to left.

37°C for 30 minutes with and without ATP and an ATP regenerating system, which consisted of phosphoenolpyruvate (PEP) and pyruvate kinase. Then the nuclei were digested with micrococcal nuclease. The amount of mononucleosome size DNA at a given level of TCA soluble DNA is about twice as high for the nuclei which were preincubated with ATP and the ATP regenerating system. This is shown in Figure 2 which compares the DNA gel pattern observed when 10 percent of the DNA was TCA soluble. In Figure 3, the slope of the line for the nuclei incubated with ATP is about twice that of the nuclei incubated without ATP. Thus the ATP incubation decreases the degradation of mononucleosomal DNA relative to the total DNA degradation.

There was variation in some experiments in the amount of mononucleosome size DNA recovered at a given level of TCA soluble DNA. Part of this variation is related to different nuclear preparations. Also a number of different scintillants and different methods for dissolving gel slices were used in early experiments. Although the slope of the line for nuclei incubated without ATP could range from 0.5 in early experiments to 2.5 in later experiments, the slope of the line for nuclei incubated with ATP was generally double that.

The difference observed in Figures 2 and 3 due to ATP could be attributed to a number of causes and not necessarily to nucleosome "unfolding" in the absence of ATP. These include: (1) increased histone degradation or H1 displacement in the absence of ATP, (2) increased nicking of DNA in the preincubation without ATP, and (3) nucleosome sliding in the absence of ATP.

Figure 3. Recovery of mononucleosome size DNA at various levels of acid soluble DNA during micrococcal nuclease digestion. Nuclei were preincubated at 37°C without (●-●) and with 2.5 mM ATP and 10 mM PEP (o-o).

If histones were degraded in the absence of ATP, more DNA would become available to micrococcal nuclease and we might expect to see the results shown in Figure 2 and 3. When histones were acid extracted from nuclei (26) after the incubation with and without ATP, and quantitated after gel electrophoresis, there was no difference in the relative amounts of core histones, and no difference in the H1/H4 ratios.

If an endogenous nuclease extensively nicked the DNA in the nuclei incubated without ATP, the starting material for the micrococcal nuclease digestion would be different even if the level of acid soluble material was approximately the same. However, there was no difference in the sedimentation profiles of the DNAs when the two types of nuclei were analyzed on alkaline sucrose gradients.

When chromatin is incubated under certain temperature and ionic strength conditions, nucleosomes can slide along the DNA, resulting in regions of densely packed nucleosomes and free DNA (27). If sliding occurred in the absence of ATP, nuclease digestion would result in a significant decrease or loss of the regularly spaced bands of nucleosome dimer, trimer, etc DNA observed in electrophoretic gel patterns. When double stranded DNAs from nuclei partially digested with micrococcal nuclease were electrophoresed, there was no significant difference between samples incubated with or without

TABLE I
The Effect of Increasing Preincubation Times
With and Without ATP on the Micrococcal Nuclease Digestion.

Time Minutes	(-) ATP	Slope (+) ATP
0	0.5	0.6
5	0.5	0.8
10	0.4	1.1
30	0.5	1.0

The preincubation at 37°C with and without ATP and the regenerating system, and the incubation with micrococcal nuclease were done as described in the text. The slope was derived as shown in Fig. 1. Gels were crosslinked with DATD, dissolved with periodic acid, and counted in Safety-Solve. Increased quenching of counts in these gel samples accounts for low slopes compared to those of later experiments.

ATP. The average repeat distance over seven nucleosomes was 192 bp for nuclei preincubated with ATP compared to 188 bp for nuclei without ATP, a difference not considered to be significant.

The ATP effect observed with nuclei was not observed with oligonucleosomes. When chromatin fragments were isolated from the nuclei as described by Butt *et al.* (23), and the polynucleosome chains were incubated at 37°C with and without ATP and the ATP regenerating system, then digested with micrococcal nuclease, there was no difference in the recovery of mononucleosome size DNA with or without ATP at the different levels of acid soluble DNA. Thus, the ATP mediated apparent stabilization of mononucleosomes occurs only with nuclei.

The nuclei must be incubated with ATP and the regenerating system for at least 10 minutes at 37°C prior to the nuclease addition in order to observe the maximum ATP mediated stabilization of nucleosomes (Table I). When ATP was added just before the nuclease, without any preincubation at 37°C, there was no significant difference in the amount of monomer length DNA released at the lower levels of acid soluble material. If the nuclei were incubated with ATP

TABLE II
Effect of Varying ATP Concentrations

Preincubation Conditions	Slope
no additions	2.3
10 mM PEP	2.4
0.10 mM ATP 10 mM PEP	2.7
0.25 mM ATP 10 mM PEP	3.6
0.5 mM ATP 10 mM PEP	3.8
2.5 mM ATP 10 mM PEP	3.9

The preincubation was for 30 minutes at 37°. The incubation was as described in the text.

and the regenerating system for 5 minutes at 37°C before the nuclease was added, some difference was observed. After 10 minutes at 37°C with ATP and the regenerating system, the difference was as great as that observed after a 30 minute preincubation.

The effect of varying the ATP concentration in the preincubation is shown in Table II. At 0.25 mM ATP the slope was 78% of that observed with 2.5 mM ATP. However, if the ATP concentration was decreased to 0.1 mM, only 26% of the original ATP effect was observed. The concentration of ATP required for the effect is similar to that required for the calf thymus topoisomerase II (28). PEP alone had no significant effect on the slope. Acetyl phosphate with acetate kinase could be substituted as a regenerating system.

ATP must be maintained in the system in order to observe the effect. If the nuclei were collected by centrifugation after a 30 minute preincubation, washed and resuspended in buffer without ATP before the nuclease digestion, no difference between the incubations with and without ATP was observed. This suggests that the ATP effect is rapidly reversible, or that the ATP effect is on the nuclease.

ATP with its regenerating system inhibits partially the digestion of free DNA by micrococcal nuclease. The rate of release of acid soluble nucleotides during the incubation of nuclei with micrococcal nuclease was inhibited approximately 50 percent with 2.5 mM ATP, and adjustments of the Ca^{++} level did not alter the inhibition.

Although ATP inhibits partially micrococcal nuclease activities, four types of experiments provide evidence against this as the explanation of the ATP effect in nuclei. The first experiment involved maintaining the same level of ATP during the micrococcal nuclease digestion of nuclei previously preincubated with or without ATP (Table III). Two preincubations were started with nuclear DNA at 3 mg/ml. One contained 2.5 mM ATP, 25 mM PEP, and 4 units/ml pyruvate kinase. After 30 minutes at 37°C, the samples were diluted ten fold and divided in half to give four samples. ATP and PEP were immediately added to one of the two ATP containing samples (Table III, sample 1) to maintain the ATP and PEP concentration at 2.5 mM and 10 mM respectively during the subsequent nuclease digestion. The slope was 2.9. No additions were made to the second sample preincubated with ATP and diluted 10 fold (sample 2). The slope was 2.8. ATP and PEP were added to one half of the sample preincubated without ATP to bring the concentrations to 0.25 mM and 2.5 mM respectively before the nuclease digestion, (sample 3) (the same level as in sample 2). The slope was 1.8. No additions were made to sample 4, the

TABLE III

Effect of Comparable Levels of ATP During the Micrococcal Nuclease Digestion.

Sample	Preincubation Conditions	Digestion Conditions	Slope
1	2.5 mM ATP 25 mM PEP	2.5 mM ATP 25 mM PEP	2.9
2	2.5 mM ATP 25 mM PEP	0.25 mM ATP 2.5 mM PEP	2.8
3	no additions	0.25 mM ATP 2.5 mM PEP	1.8
4	no additions	no additions	1.4

The preincubations and incubations were as noted in Table II.

control, and the slope was 1.4. The increase of sample 3 is 29 percent of the increase of sample 2 above the base line value (sample 4), and not 100% as expected if all of the ATP effect were on the micrococcal nuclease. The time of manipulation in the presence of ATP may be responsible for the slope of 1.8 rather than 1.4. The second type of experiment supporting the concept that the ATP effect is not on the micrococcal nuclease was shown in Table I and demonstrated that preincubation was required. The third type of experi-

Figure 4. Effect of novobiocin on the recovery of mononucleosome size DNA at various levels of acid soluble DNA during micrococcal nuclease digestion of nuclei preincubated at 37°C without (●-●) and with (○-○) 2.5 mM ATP and 10 mM PEP. (a) No novobiocin. (b) 0.5 mM novobiocin. (c) 1 mM novobiocin. (d) 1.5 mM novobiocin. (e) 2 mM novobiocin. (f) 2 mM novobiocin and 5 mM ATP.

TABLE IV
The Effect of Topoisomerase II Inhibitors on Mononucleosome DNA Degradation

	Slope		
	Control	ATP 2.5 mM	ATP 5 mM
A. Novobiocin (mM)			
0	2.5	4.8	-
0.5	2.5	4.1	-
1.0	2.7	3.4	-
1.5	2.8	3.0	-
2.0	2.8	2.7	-
2.0	2.5	-	4.4
B. VM-26 (μg/ml)			
0	2.4	4.7	
50, just before ATP	1.7	4.1	
50, 5 min before ATP	2.1	4.2	

The preincubations and incubations were as noted in Table II.

ment involved the absence of an ATP effect when isolated chromatin fragments were digested. The fourth type of experiment involved the absence of a ATP effect in nuclei from log phase cells as will be discussed. These are four lines of evidence against the possibility that the ATP effect is due to nuclease inhibition.

Inhibitors

When novobiocin was added to the nuclei incubated with and without ATP, the ATP effect was eliminated. This is shown in Figure 5 and Table IV. The extent of inhibition was proportional to the novobiocin concentration. A typical ATP mediated protection of nucleosomes due to 2.5 mM ATP was gradually lost as the novobiocin concentration was increased from 0.5 mM to 1.5 mM. With 1.5 mM and 2 mM novobiocin, there was no significant difference between the control and the ATP containing nuclei. The inhibition by 2 mM novobiocin could be partially reversed by increasing the ATP concentration to 5 mM.

The inhibition by novobiocin plus the absence of an ATP effect with isolated chromatin fragments suggested that DNA supercoiling and torsional stress produced by a topoisomerase II activity might be responsible for the ATP mediated stabilization of nucleosomes. Nalidixic acid (0.5 and 2 mg/ml), coumermycin (0.5, 2.0 mM), and oxolonic acid (100, 400 μg/ml) did not inhibit the ATP effect. Although these drugs are strong inhibitors of *E. coli* gyrase, they are only weak inhibitors of HeLa topoisomerase II (L. Liu, personal communication). The lack of inhibition by these drugs did not rule out the possibility that HeLa topoisomerase II is involved. VM26 or tenoposide is a

TABLE V
Effect of Nucleotides on Mononucleosome Degradation

	<u>Slope</u>	<u>Percent of Increase with ATP</u>
control	2.4	-
ATP	4.7	100
dATP	4.9	109
TTP	4.5	91
dGTP	4.1	74
dCTP	4.1	74
CTP	3.6	52
UTP	3.5	48
GTP	3.3	39

The preincubations and incubations were as noted in Table II. The slopes represent the averages of two experiments.

potent inhibitor of Hela topoisomerase II. It traps the enzyme in a cleavable complex where the ends of the DNA are not free to rotate (29), a mechanism different from that of novobiocin. VM26 at a concentration of 50 $\mu\text{g/ml}$ did not significantly inhibit the ATP mediated stabilization of nucleosomes (Table IV).

Novobiocin inhibits not only topoisomerase II but also eukaryotic polymerase α (30). Aphidicolin, which is a specific inhibitor of eukaryotic polymerase α activity, did not at a concentration of 5 $\mu\text{g/ml}$ eliminate the ATP effect. This makes is unlikely that novobiocin is acting on polymerase α . Also the four deoxynucleoside triphosphates at 0.1 mM did not reproduce the ATP effect.

Nucleotide requirements

The ATP mediated protection of nucleosomes was not specific for ATP (Table V). dATP could substitute for ATP. All the other nucleoside triphosphates tested were also able to stabilize nucleosomes to varying extents with TTP being the most effective while UTP and GTP were least effective (Table V). This is in contrast to Hela topoisomerase II which is specific for ATP and dATP and will not work with GTP, CTP, and UTP (31). Neither cAMP nor cGMP stimulated the effect of ATP when added to the nuclei at a final concentration of 10 μM . It is unlikely that these nucleoside triphosphates are acting by regenerating residual ATP in the nuclei since the level of ATP was 0.009 nM/ 10^6 nuclei as opposed to 5.5 nM of ATP/ 10^6 whole cells.

The results with ATP analogs were highly variable. The thiophosphate derivative, ATP γ S, was able to substitute for ATP in one experiment, but not in a second. The methylene derivatives also varied in their ability to substitute for ATP from one experiment to the next. When the results of seven experiments were averaged, AMPCPP was 73% as effective as ATP in protecting

TABLE VI
Effect of Cell Growth and of 2-Deoxyglucose
on the Degradation of Mononucleosomes

Exp	Conditions	Slope	
		Control	+ATP
1	Non-Proliferating	2.0	3.5
	Proliferating	3.0	3.0
2	Proliferating	2.9	3.0
	Proliferating plus 2-Deoxyglucose	2.4	5.5

Proliferating cells were labelled and isolated while in log phase growth. Non-proliferating cells were in a stationary phase during the period of thymidine incorporation.

nucleosomes. And from the results of nine experiments, AMPPCP was 35% as effective as ATP. Thus, while binding of a nucleotide residue apparently produces some protection, the ability to act as a phosphate donor may enhance the ATP effect. A similar situation has been observed with the ATP analogue (β , γ -imido) ATP which can trigger one cycle of supercoiling with the pro-caryotic DNA topoisomerase II (32).

Reproducibility of nuclear preparations

The ability to respond to ATP varied not only from one batch of nuclei to the next, but also with specific preparations of nuclei, depending on the time of storage. The nuclei were stored at -20°C in 50% glycerol. Aliquots of some preparations of nuclei could be used for up to three weeks after the date of preparation and still give the same ATP effect as was observed immediately after preparation. Often, however, the nuclei failed to respond to ATP after being stored at -20°C for longer than a week. Therefore, nuclei were routinely used within a week after preparation.

The ability to respond to ATP also depended on the state of proliferation of the cells from which the nuclei were isolated (Table VI, Experiment 1). Cells which were actively dividing and where the cell number had almost doubled in the 24 hours before harvesting did not show an ATP effect, while cells which were not dividing and where the cell number had not increased significantly in the 24 hours preceeding harvesting responded to ATP.

The ATP level in the perchloric acid extract of non-proliferating cells was approximately 60% of the level in proliferating cells. An experiment to lower the level of ATP in proliferating cells was done. An aliquot of proliferating Hela cells was exposed in Joklik's medium to 2-deoxyglucose (6 gm/l) for 30 minutes at 37°C before harvesting and preparation of nuclei. This treatment decreased the intracellular ATP level from $5.5 \text{ nM}/10^6$ cells to 1.5

nM/10⁶ cells or to a level of 27% of that in the untreated cells. As shown in Table VI, Experiment 2, the untreated proliferating cells showed no ATP effect while the deoxyglucose treated cells did show an ATP effect. Note that the slope of the control proliferating cells with deoxyglucose is lower than that of cells without. These experiments suggest that in order to see the ATP effect, it is necessary for the energy producing systems to be altered from those in the proliferating cells. Chromatin may be more "relaxed" in this state and mononucleosomes more open for degradation, a state reversed by incubation with ATP.

Miscellaneous

DTT was required to obtain the ATP effect whereas PMSF could be omitted without noticeably changing the results. The ATP effect was not stimulated by the addition of 0.1 mM NAD, and 5 mM 3-amino-benzamide was not inhibitory. NAD alone had no effect. KFl at 10 mM did not alter the ATP effect.

DISCUSSION

In the absence of ATP, nucleosomes in nuclei from nonproliferating cells undergo a conformational change which results in an increased relative sensitivity to micrococcal nuclease of the DNA of mononucleosomes relative to DNA made acid soluble. When ATP and an ATP regenerating system are included in a 30 minute incubation at 37°C before nuclease digestion, about twice as much DNA is recovered in mononucleosome size fragments at the same level of acid soluble material as when the ATP is omitted. This ATP effect results in a relative stabilization of mononucleosomes due either to a decrease in the degradation of mononucleosomal DNA relative to DNA made acid soluble, or to a decrease in the amount of DNA made acid soluble relative to that converted to mononucleosomes. We are unable to distinguish between the two possibilities by measuring absolute rates since ATP partially inhibits the rates of both processes. The conformational change due to ATP cannot be attributed to increased histone or DNA degradation, or to nucleosome sliding in the 30 minute incubation at 37°C without ATP. The ATP effect is also not due to partial inhibition of nuclease activities for the four reasons outlined in the results section.

The reversible inhibition of the ATP effect by novobiocin suggested that some action on DNA topology by topoisomerase II might be responsible for the ATP effect. HeLa cell topoisomerase II is inhibited 50% by 200 µg/ml of novobiocin (0.33 mM) (31). Novobiocin has been shown to affect DNA repair (33-36), minichromosome formation (37), and preferential DNase sensitivity of

β -globin genes (38). However, evidence against topoisomerase II as responsible for the ATP effect is the inability of VM-26 to block the ATP effect as well as the ability of all of the ribo- and deoxyribonucleoside triphosphates to reproduce to varying degrees the ATP effect.

The ability of the ATP incubation to protect mononucleosome DNA while linker DNA was being degraded suggests that the ATP effect could involve H-1 histone. This histone binds to the core particle at the entrance and exit of the DNA (1,2). On digestion of chromatin DNA with micrococcal nuclease, there are pauses at approximately 165 and 145 bp of DNA (39). The chromatosome which contains approximately 165 bp of DNA, the histone octamer and one molecule of histone H-1, can be isolated after a partial micrococcal nuclease digestion (40). If the histone H-1 is removed from chromatin, a core particle containing DNA of approximately 145 bp, rather than 165 bp, is isolated after micrococcal nuclease digestion. If, in our experiments, ATP increased the binding of H-1, we would expect to see in the mononucleosome DNA, a shift in size from 145 to 165 bp. Such a shift is apparent in Figure 2 but this shift was not a consistent finding in all experiments where a significant ATP effect was seen. Thus, we have no solid evidence that this is the mechanism by which ATP protects mononucleosomes during the micrococcal nuclease digestion. It is possible that ATP helps to preserve a superstructure which is histone H-1 dependent (41) and which is sensitive to novobiocin.

From a 3.3 Å resolution x-ray crystallographic map of the histone core, Burlingame *et al.* (4) suggest that "the nuclease sensitivity of the DNA in active genes is the result of a relaxed DNA structure caused by the loss of contact of the histone dimer (H2A-H2B) with the tetramer" (H3-H4). The dimers are loosely bound, as *in vitro* exchange of H2A and H2B has been noted (42). This type of change we would not recognize in our examination of the levels of the histone core proteins.

It is possible that nucleoside triphosphates bind to histones in channels in the core particle (4), and this binding affects the DNA-histone association. Of interest is the observation by Cotton *et al.* (43) that novobiocin precipitates purified histones by interaction with arginine residues. Although these authors were unable to demonstrate any effect of ATP on this precipitation, it is possible that competition between ATP and novobiocin for binding sites in nucleosomes could occur.

Finally the ATP effect is observed in nuclei only under certain conditions. It is absent in cells in log phase, it can be regained if the ATP stores of log phase cells are depleted, and it is lost from nuclei upon

prolonged storage. It is as though the chromosomal structures undergo some "relaxation" process which for a period of time is reversible by incubation with ATP, but eventually becomes irreversible.

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