DNA Internal Motions within Nucleosomes during the Cell Cycle and as a Function of Ionic Strength

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ABSTRACT: We have used ^{31}P NMR spectra to show that DNA internal motions are greatly hindered within oligonucleosomes. The fluctuations seem to be a function of both the cell cycle and the number of nucleosomes interlinked. Namely, the resonance areas, directly related to unbound phosphate, are consistently smaller in M-phase than in S-phase; at the same time, the resonance line width, inversely related to base plane, deoxyribose, and phosphate internal motions, is consistently larger in mononucleosomes than in oligonucleosomes. In all cases, the removal of chromosomal proteins, by a progressive increase of ionic strength up to 2 M NaCl, increases the internal motion, as monitored by a decrease in line width toward that of free DNA. While for both oligo- and mononucleosomes in S-phase the decrease in line width is strictly correlated to a sharp increase in resonance area, in M-phase it is not, with the ^{31}P resonance area rather low even at 2.0 M NaCl extraction. Similarly, while S-phase ^{31}P line widths steadily grow from mono- to oligonucleosomes, in M-phase they do not. Moreover, the increase of the ionic strength to 0.6 M NaCl, as compared to 0.35, 1.2, and 2 M NaCl, displays significant variations on ^{31}P line width and resonance area, independent of the cell cycle phase and the number of nucleosomes interlinked. These observations agree with earlier suggestions on the differential role of the various chromosomal protein subfractions, known to preferentially dissociate at the different ionic strengths in question, in the sealing of mononucleosomes and in the overall stability of polynucleosomes.

Nuclear magnetic resonance studies of long DNA fragments have shown that the internal structure of B-form DNA is not rigid but it experiences large fluctuations in nucleotide conformation which occur with a time constant near 10^{-9} s (Hogan & Jardetzky, 1979; Bolton & James, 1980; Klevan et al., 1979; Early & Kearns, 1979). Namely, from ^{1}H and ^{31}P NMR relaxation and line-width measurements, the B-DNA helix turns out to experience large fluctuations in the conformation of the base and sugar, as well as the deoxyribose–phosphate backbone.

It has been reported that, when ethidium bromide (EB) intercalates into a 300 bp long DNA, the ^{31}P NMR resonance area becomes unmeasurable within a 2 bp long region and that the relaxation properties of DNA out of the binding site are nearly unaffected by bound EB. From these observations, Hogan and Jardetzky (1980) came to the conclusion that base plane, deoxyribose, and phosphate backbone internal motions are "frozen out" within the 2 bp long EB–DNA complex but that DNA regions immediately adjacent to the complex are nearly unaffected.

Similarly, following earlier studies on the interaction of DNA with H1 histone (Dolby et al., 1981; Bradbury & Baldwin, 1986; Belmont & Nicolini, 1981) and HMG non-histone (Weisbrod & Weintraub, 1979) chromosomal proteins, in the present work we have explored the role of the above proteins in the control of DNA internal motions within nucleosomes. Using ^{31}P NMR spectroscopy, we have measured resonance area and line-width changes, related to unbound phosphate backbone and internal motions, which occur when nucleosomes, isolated from HeLa cells synchronized in different phases of the cell cyle or logarithmically growing, are progressively exposed to increasing NaCl concentrations previously associated with the selective and successive removal of HMG non-histone and H1, H2A–H2B, and H3–H4 histones (Dolby et al., 1981; Bradbury & Baldwin, 1985; Weisbrod & Weintroub, 1979; Wachtel et al., 1981).

MATERIALS AND METHODS

Nucleosomes were isolated by a standard procedure from HeLa cells. Namely, culture of HeLa cells and nuclei isolation were done according to Yau et al. (1982). Isolated nuclei suspended in 10 mM Tris-HCl, pH 6.85, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.25 M sucrose, and 0.2 mM PMSF at 2 mg/mL (DNA) were incubated previously at 37 °C for 15 min. Digestion of nuclei was started by adding micrococcal nuclease (Worthington) at 25 units/mg (DNA) for 15 min with continuous shaking. The reaction was terminated by adding 20 mM EDTA to a final concentration of 2 mM. HeLa cells were blocked in mitosis by 0.06 μg/mL colcemide (M-phase) or blocked at the beginning of DNA synthesis with hydroxyurea (S-phase).

Digested nuclei were spun down, resuspended in lysis buffer (10 mM Tris-HCl, pH 6.85, 5 mM EDTA, and 0.1 mM PMSF), left on ice for 15 min, and spun at 5000*g* for 10 min to remove nuclear debris. Then clarified and solubilized chromatin was applied to Beckman SW 27 linear 5–20%

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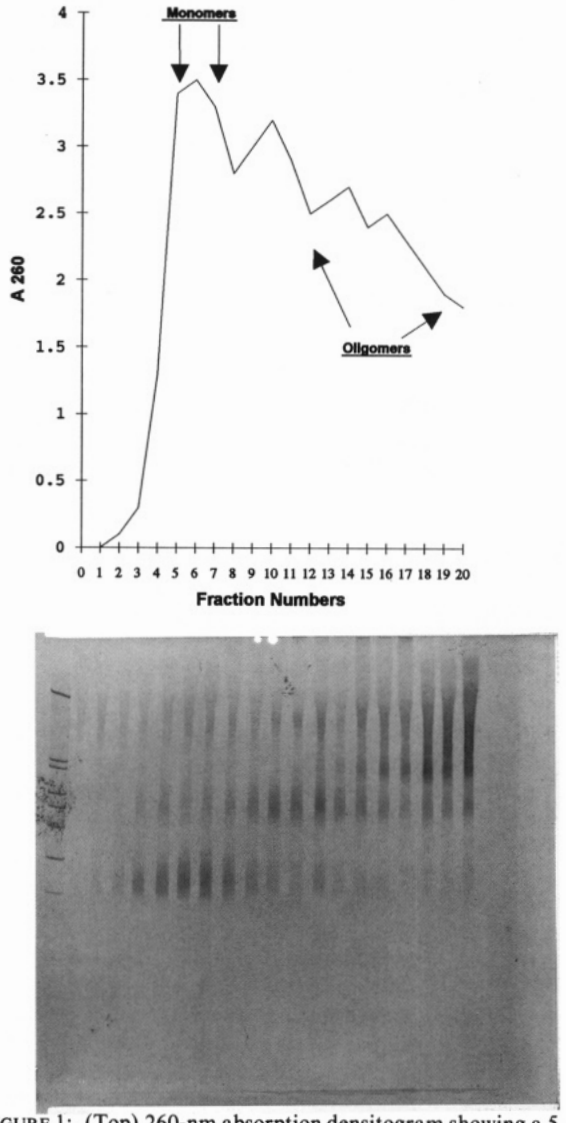

FIGURE 1: (Top) 260-nm absorption densitogram showing a 5–20% sucrose gradient of HeLa cells treated with colcemid and thereby blocked in M-phase. (Bottom) Polyacrylamide DNA gel showing the last 20 fractions of the same sucrose gradient. The last lane on the right represents linear DNA markers of 154, 220, 298, 344, 396, 506, 517, and 1631 bp.

sucrose gradients containing lysis buffer and spun at 25 000 rpm at 4 °C for 20 h. Fractions from sucrose gradients containing either mononucleosomes or oligonucleosomes were pooled (Figure 1, top). Figure 1 (top) shows how oligonucleosomes are in fact a mixture of polynucleosomes of different length, i.e., from tri- to esanucleosomes. DNA gel analysis, from corresponding fractions, indicated the sample was free from degradation and contained oligonucleosomes of the expected length (Figure 1, bottom), i.e., mononucleosomes and oligonucleosomes. DNA gel analysis also showed that the oligonucleosome samples from both hydroxyurea- and colcemide-treated HeLa cells had a similar, but not identical, length in terms of the number of nucleosomes interlinked.

Samples were then dialyzed several times, to remove the sucrose, against a buffer consisting of 10 mM Tris-HCl, pH 7.0, and 2 mM EDTA. Trimethyl phosphate (TMP) was then added as an external standard. ^{31}P NMR spectra were acquired at 22 °C on a 300-MHz GE Omega spectrometer and on a 500-MHz Bruker AM. With a sample size of 2 mL the concentration of oligonucleosomes was typically 2 mg/mL and the number of scans 1024. The spectral width was set at 5000 Hz; 512 data points were acquired for an acquisition time of 0.1 s. The recovery delay was 2 s. During the processing, FIDs were apodized with a line broadening of 20 Hz to improve the signal to noise ratio and zero-filled to 8K data points with a final digital resolution of 0.6 Hz/point. Line broadening was taken in consideration in the line-width and area resonance calculations. In all cases, the delay between acquisitions was sufficiently long so that a further delay did not increase the area under the resonance line.

RESULTS

Figure 2A,B shows the effect of NaCl concentration on nucleosomes in S-phase. The peak on the left centered at 0 Hz is the TMP used here as external reference. Almost all the broadening of the TMP peak is due to the strong line broadening of the exponential multiplication necessary to improve as much as possible the sensitivity of our spectra. All the experiments were run at room temperature. Above 20 °C, the effect of the chemical shift on DNA phosphates is much smaller than the line-width contribution and it can therefore be ignored.

In every phase it is possible to observe a broadening of the line when mononucleosomes are compared (Figure 2A) with oligonucleosomes (Figure 2B) at each ionic strength. In Table I, the results of line-width measurements for the different samples are shown. The first effect we notice is the monotonic decrease of the line width with increasing [NaCl]. The narrowing is due to an increase of the T_2 time together with progressive chromosomal protein removal. There are at least two reasons for the change in line widths: (1) an increase in the internal motions of the DNA, due to the number of free phosphates left unbound by protein removal; (2) an increase of the correlation time, related to the overall rotational tumbling of the nucleosomes in solution and due to a progressive uncoiling of the DNA resulting from the loss of structure caused by the removal of chromosomal proteins.

Point 1 would lead to a narrowing of the line width whereas point 2 would lead to a broadening of the line width. The decrease in the overall line width by chromosomal protein removal points out the predominance of the effect due to the internal motions with respect to the opposite effect due to a loss of structure. We should also mention the T_2 artifacts brought by the strong ionic concentration. Such an effect appears unnoticeable, however, considering that the line-width of the TMP used as external reference remains constant at the different ionic strengths (see Figure 2).

In Table I, M-phase oligonucleosome half-widths at half-height (HWHH) appear much narrower when compared to S- and log-phase while the differences at each ionic strength are minimal for mononucleosomes.

If the line width is a measurement of the degree of motion of DNA phosphates, the resonance peak area can be considered as a rough estimation of the number of free phosphates of the molecule. Even though the binding of histones to DNA is not as strong as the ethidium bromide binding that "freezes out" a 2 bp long DNA region adjacent to the binding site, we should expect, however, an increase of the resonance area with protein removal due to the higher number of free phosphates in the nucleosomal DNA backbone.

Going from 0 to 2 M NaCl, a loss of sensitivity was apparent, likely due to the worsening of the field homogeneity with increasing ionic molarity of the solution. The DNA phosphate resonance areas were therefore normalized with respect to the TMP resonance area (Table II).

If we make the conservative assumption that complete chromosomal protein removal occurs at 2.0 M NaCl, the

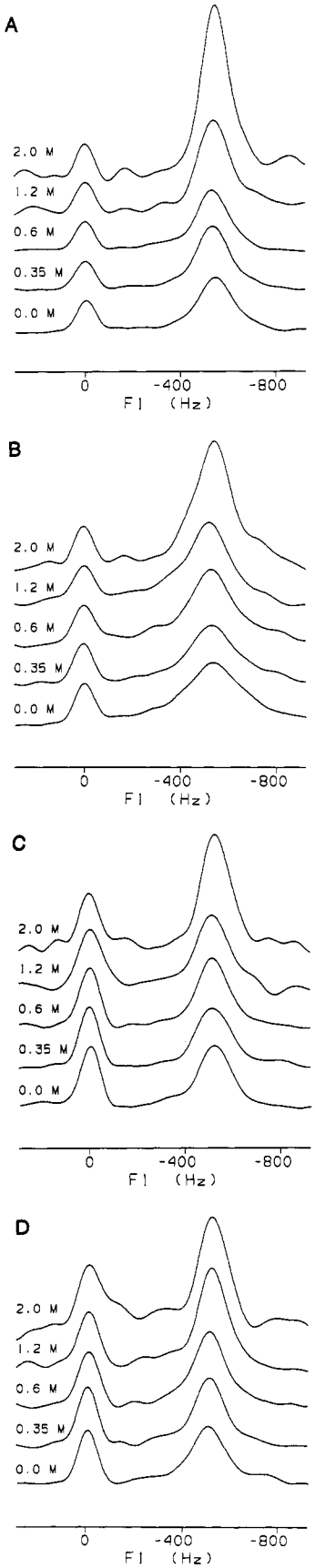

FIGURE 2: ^{31}P NMR spectra of S-phase (A) mononucleosomes and (B) oligonucleosomes at different NaCl concentrations. (C) and (D) are the same as (A) and (B) for nucleosomes in M-phase. The samples were in 10 mM Tris-HCl, pH 7.0, and 2 mM EDTA. The external standard is TMP and the temperature 22 °C. 1024 scans were collected for an acquisition time of 0.1 s on a spectral width of 5000 Hz. The recovery delay was 2 s. All the spectra were calibrated with respect to the TMP peak.

Table I: ^{31}P NMR Half-Width at Half-Height (HWHH) Values of Oligonucleosomes in S-, log-, and M-Phase versus Salt Concentration in 10 mM Tris-HCl, pH 7.0

	monomers			oligomers		
[NaCl]	S-phase	log-phase	M-phase	S-phase	log-phase	M-phase
0 mM	170	164	159	280	222	177
350 mM	162	163	155	243	204	156
600 mM	160	153	148	226	181	154
1.2 M	148	146	140	217	160	136
2.0 M	124	120	133	180	156	128

Table II: ^{31}P NMR Resonance Area of Oligonucleosomes in S- and M-Phase versus Salt Concentration in 10 mM Tris-HCl, pH 7.0[a]

	S-phase		M-phase	
[NaCl]	monomers	oligomers	monomers	oligomers
0 mM	3.6	3.9	1.9	2.4
350 mM	4.7	3.8	1.9	2.5
600 mM	5.4	4.7	1.8	2.6
1.2 M	5.9	5.4	2.1	2.9
2.0 M	8.0	11.5	2.7	3.3

[a] The normalized area was obtained by dividing the nucleosome area by the TMP area, calculated by integrating the peaks at each salt concentration from Figure 3. The numbers are expressed in arbitrary units.

fraction (F_M) of bound phosphates, defined as $F = 1 - (A_R/A_{2.0})$, can be plotted against the NaCl molar concentration for both mono- and oligonucleosomes, where $A_{2.0}$ is the resonance area at 2.0 M NaCl (Figure 3). The maximum value of F_M reached at 10 mM Tris-HCl is proportional to the fraction of ^{31}P resonance area which can be lost in the native complex by chromosomal protein binding. From Figure 3, it is also possible to understand the effect of different ionic strengths on overall nucleosome stability.

When we consider mononucleosomes in S-phase (Figure 2A), we can see a noticeable effect at 0.35 and 0.6 M NaCl, little effect at 1.2 M NaCl, and again significant changes at 2.0 M NaCl (Figure 3A). On the other hand, oligonucleosomes (Figure 3B) still behave in the same way, but the effect at 0.35 M NaCl disappeared.

When compared with S-phase, M-phase mononucleosomes (Figure 2C) appear to be similar in line widths (Table I). Such similarities end when we consider oligonucleosome salt-induced line widths. F_M values for mononucleosomes and oligonucleosomes in M-phase point out very little effect at 0.35 and 0.6 M NaCl when compared to S-phase nucleosomes.

Moreover, the fraction of bound phosphate for NaCl concentrations lower than 1.2 M seems to be much smaller in M-phase than in S-phase (Figure 3A,B). From Figure 3, it is evident how the change in F_M is much smaller in M-phase than in S-phase. Such an effect is even worse if we consider either F_M. Figure 4A) or resonance area (Figure 4B) versus HWHH, being much sharper in the latter for S-phase than for M-phase. However, even after complete protein removal at 2 M NaCl, the HWHH (Table I) never approaches the rather low value for free DNA of equivalent length (not shown).

CONCLUSIONS

In this paper, we have shown that DNA internal motion is highly and selectively reduced within nucleosomes by the binding of chromosomal proteins and it is a function of cell cycle progression (M- versus S-phase, versus log-phase).

Due to protein binding, the amplitude of DNA motions appears reduced, and the rate of motions is significantly slower: therefore, DNA regions at the binding sites, and in

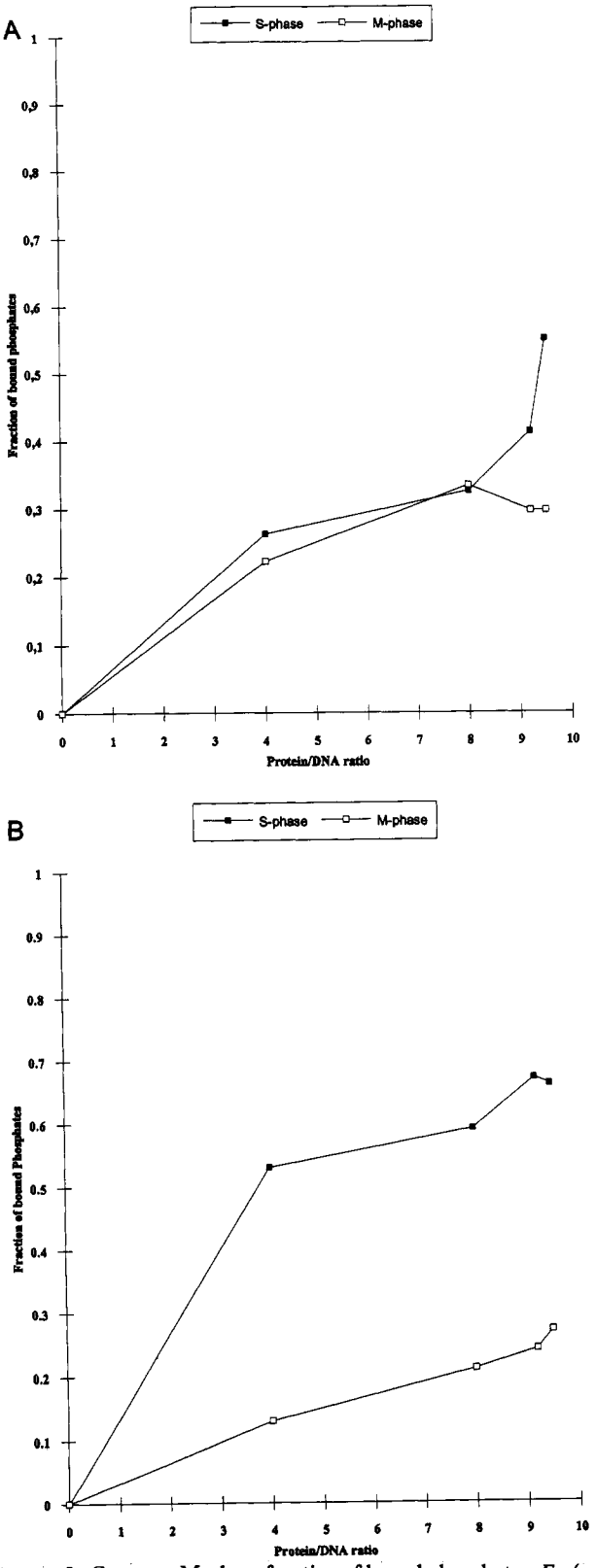

FIGURE 3: S- versus M-phase fraction of bound phosphates, F_M (see text for details), plotted against NaCl molar concentration for (A) mononucleosomes and (B) oligonucleosomes. $F_{2.0}$ assumes complete chromosomal protein removal at 2.0 M NaCl. Note that the first experimental point is taken at 10 mM Tris-HCl buffer.

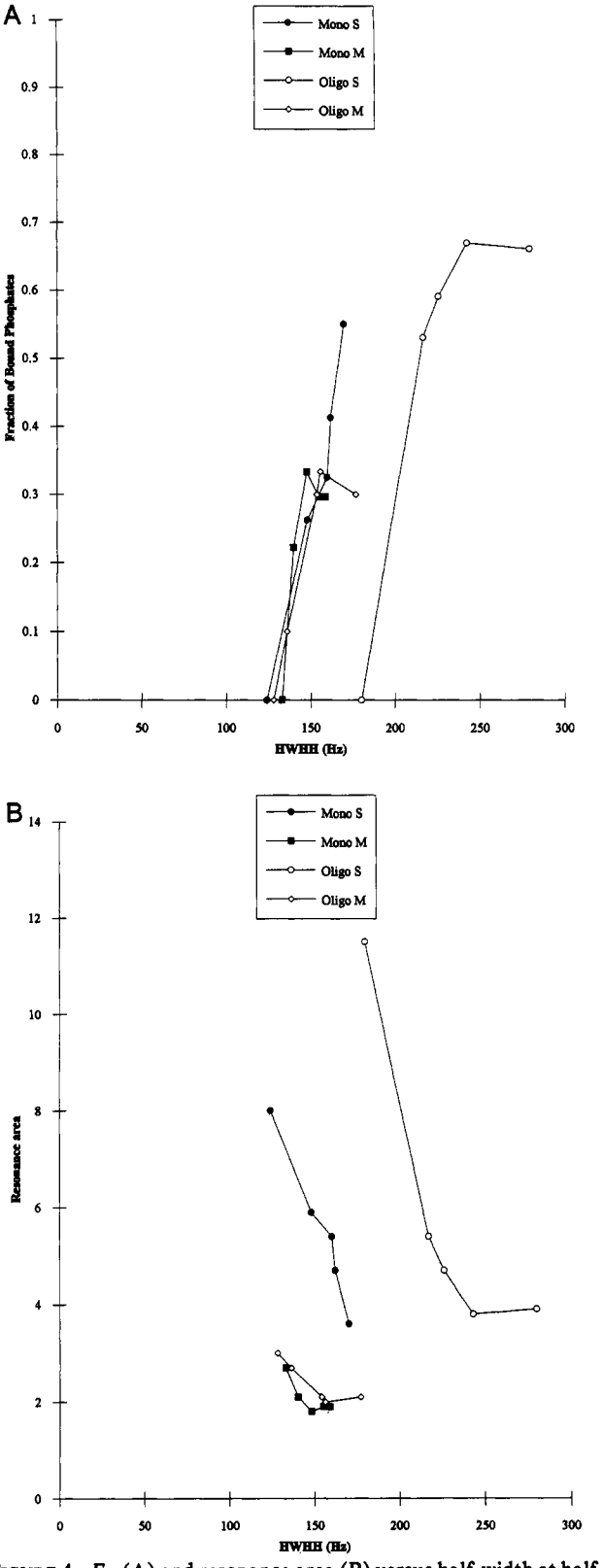

FIGURE 4: F_M (A) and resonance area (B) versus half-width at half-height (HWHH) at different NaCl molar concentrations for different nucleosome lengths and cell cycle phases.

their proximity, behave as rigid; i.e., ^{31}P NMR spectra of bound DNA regions become unmeasurable in solution.

Oligonucleosomes in mitosis have a much smaller correlation time; i.e., nucleosome–nucleosome interaction is much stronger here, stabilizing the fiber in a much more compact structure characterized by a longer T_2 relaxation time. However, this effect is not visible on mononucleosomes where, obviously, no nucleosome–nucleosome interaction is present.

The stoichiometry of phosphorus area loss is more complicated, but appears to be due, as previously suggested (Hogan & Jardetzky, 1980), to the coupling between phosphorus and deoxyribose motions. In the case of both S-phase and M-phase, on raising the ionic strength significant variations occur on

^{31}P line-widths and resonance areas, pointing to reduced relaxation properties of DNA in M-phase.

log-phase nucleosomes show an intermediate behavior between S-phase and M-phase (Table I), as expected. log-phase nucleosomes are compared of a mixture of different phases of the cell cylce, mostly G1-phase.

The lower fraction of bound phosphates in M-phase, when compared to S-phase, points out the smaller affinity of core histones in the binding of the phosphate DNA backbone during mitosis, sometimes associated with the observed H4 acetylation during mitosis (Alfrey et al., 1964).

The consistently smaller line-widths for M-phase oligonucleosomes with respect to S-phase oligonucleosomes is remarkable.

Indeed, the overall ^{31}P spectra ionic strength dependence is compatible with earlier suggestions on the differential role of the various chromosomal subfractions in the sealing of mononucleosomes and in the stability of polynucleosomes.

Many publications during the past 15 years have suggested that HMG proteins come off at 0.35 M NaCl, H1 at 0.6 M NaCl, H2A/H2B at 1.2 M NaCl, and H3/H4 completely off at 2.0 M NaCl (Wachtel et al., 1981). However, it might not be invariably the case that the above clean separation of chromosomal proteins can be achieved by raising the ionic strength from 0.35 to 0.6, 1.2, and 2 M NaCl. Similarly, it is not known whether the histones and non-histones are fully dissociated at the ionic strength in question or simply less tightly bound, allowing their removal and exchange.

Further necessary experiments are in progress to address the above questions in detail (unpublished results).

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