# Liquid Crystalline Ordering of Nucleosome Core Particles under Macromolecular Crowding Conditions: Evidence for a Discotic Columnar Hexagonal Phase

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ABSTRACT Macromolecular crowding conditions occurring inside the cell nucleus were reproduced experimentally with solutions of mononucleosome core particles to study their supramolecular organization. We report here that under these conditions, and over a large range of monovalent salt concentrations, mononucleosome core particles self-assemble to form a discotic liquid crystalline phase characterized in polarizing and freeze-fracture electron microscopy. Mononucleosomes are stacked on each other to form columns, which are themselves closely packed into an hexagonal array. The nucleosome concentration, estimated from the network parameters, falls in the range of values measured in cell nuclei. We suggest that these concentrated solutions, although their organization cannot be immediately compared to the organization of chromatin in vivo, may be used to investigate the nucleosome-nucleosome interactions. Furthermore, this approach may be complexified to take into account the complexity of the eucaryotic chromatin.

## INTRODUCTION

In eukaryotic cells, the nuclear DNA is highly compacted through its association with histone proteins. At the first level of organization, nucleosomes are formed by coiling of the DNA molecule around a protein core. The structure of the histone octamer is a left-handed proteinaceous superhelix 6.5 nm in diameter that serves as a spool around which 146 bp of right-handed DNA is wrapped to form the nucleosome core particle, which can be described as a wedge-shaped cylinder 10.5 nm in diameter and 6 nm in height (Arents et al., 1991). Although the structure of histone octamers and nucleosome core particles is now well documented and understood (Richmond et al., 1988; Arents et al., 1991; Arents and Moudrianakis, 1993; Pruss et al., 1996), their higher order organization remains largely unknown, despite extensive research in this field (Widom, 1989; Van Holde and Zlatanova, 1995). Attempts of chromatin structure elucidation rely for a large part on experiments involving progressive decondensation of native chromatin, which unavoidably destroys the higher order chromatin organization. Other approaches consist of in vitro chromatin reconstruction from isolated polynucleosome chains condensed with cations and/or H1 proteins. However, both approaches take place in dilute media, disregarding the fact that chromatin inside the cell nucleus is a crowded medium (Fulton, 1982; Cayley et al., 1991). With the exception of the early works of Luzzati and Nicolaieff (1959, 1963) and Sperling and Klug (1977), performed in concentrated solutions, the behavior of the constitutive elements of eucaryotic chromatin under conditions of macromolecular crowding like those in living cells has not been considered. Using currently available methods to prepare large amounts of biochemically well-defined chromatin elements, we investigate the behavior of mononucleosome core particles in concentrated solution and show that they self-organize into a liquid crystalline phase. Both the limitations and promises of this model, which could evolve toward more complex systems, are discussed.

## MATERIALS AND METHODS

Large amounts of nucleosome core particles (~1 g) were prepared by micrococcal nuclease digestion of H1-depleted calf thymus chromatin. The particles, suspended in 150 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 7.0), were purified by gel filtration over a Sephacryl S300 HR column (Pharmacia), and the mononucleosome peak was collected. After the different fractions obtained by chromatography were pooled, retardation gel electrophoresis was performed on 7.5% acrylamide gels to check the integrity of the nucleosome cores and the absence of polynucleosomes as well as free naked DNA in solution (Fig. 1). The histone composition was also checked by sodium dodecyl sulfate-15% acrylamide electrophoresis.

Nucleosome core particles suspended in 100 or 150 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 7.0) were concentrated up to ~200 mg/ml by ultrafiltration in a pressurized cell throughout a hydroxypropylcellulose membrane (Amicon YM100). Further concentration (up to 350 to >400 mg/ml) was performed on 10–20-$\mu$l samples, either by slow evaporation of the solvent or by the addition of 1–2 $\mu$l of a solution of polyethylene glycol (MW 8000, 400 mg/ml in 100 mM or 150 mM NaCl). In the latter case, a phase segregation occurs and the concentrated nucleosome phase is recovered in the pellet after centrifugation (10 min, 11,000 × $g$).

A drop of the concentrated solution was deposited between slide and coverslip and observed in a Nikon Optiphot X Pol microscope. The slide and coverslip were either sealed to avoid water evaporation or left unsealed to follow the evolution of the preparation with increasing concentration. Observations were made between linear or circular crossed polarizers, and a wave plate ($\lambda$) was sometimes added to analyze the molecular orientations.

For electron microscopy, drops of the nucleosome core solution, concentrated either by dehydration or by the addition of polyethylene glycol, were deposited on gold discs and allowed to stabilize under humid atmosphere for 30–90 min. To control the liquid crystalline state of the speci-

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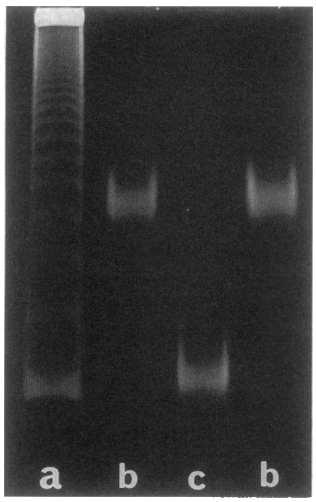


FIGURE 1 Polyacrylamide retardation gel electrophoretic pattern of the nucleosome core samples checking the absence of oligonucleosomes or free naked DNA in the solution. (*Lane a*) Standard 123-bp ladder DNA. (*Lane b*) Sample. (*Lane c*) 146-bp DNA fragments extracted from the nucleosome sample.

mens, drops of the same solutions were deposited under the same conditions on hydrophobic glass slides and observed in parallel in polarized-light microscopy. Samples were then quickly frozen by projection onto a copper bloc cooled down to 10 K with liquid helium (Cryovacublock, Reichert), and immediately transferred into liquid nitrogen. Freeze fracture was performed at 163 K under a $2 \times 10^{-7}$ torr vacuum in a Balzers BAF 400T apparatus. Fracture surfaces were etched for 2 min at 153 K, platinum-carbon shadowed at an angle of 45°, and carbon coated. Replicas were reheated to room temperature, washed in distilled water, and observed in a Philips 400 TEM at 80-kV accelerating voltage.

## RESULTS

### Liquid crystalline ordering of the mononucleosome core particles

Whatever the concentration method used, the nucleosome core particles spontaneously organize into germs of an ordered phase that is fluid, although highly viscous, as can be checked by putting gentle pressure on the preparation. Between crossed polarizers, these germs may be either nearly extinguished or highly illuminated, depending on their orientation in the preparation.

1. Highly birefringent germs are rod-shaped (Fig. 2 *a*) or may show a bilateral symmetry (Fig. 2 *c*). Assuming that the optical properties of the germs are due to the intrinsic negative birefringence of the DNA molecule and not to the histone core, we determined with the use of a λ plate inserted at an angle of 45° between crossed polarizers that the DNA molecules, on average, lie normal to the elongation axis of the germs. With the DNA wrapped around the cylindrical particles, the nucleosome core particles are thus oriented with their diameter normal to the axis of the germs.

2. Germs with a hexagonal symmetry (Fig. 2, *a* and *b*) show no or weak birefringence and are more conveniently examined between parallel polars or by phase-contrast microscopy. They grow into flower-shaped domains (Fig. 2 *b*), and dendritic ramifications can form from each branch of the germ. Between crossed polars, the extinguished germ can be illuminated by gently sliding the coverslip, thus tilting the germs more or less obliquely to the preparation plane, with deformations. The homeotropic anchoring is thus suppressed by the shear, and birefringence properties of the solution can then be seen.

When the concentration of the solution increases, more and more germs grow in the solution and finally fill the entire preparation. Yet they never merge into a homogeneous phase, but form a mosaic of juxtaposed finite-sized liquid crystalline domains ranging from a few microns to more than 50 μm.

These observations reveal a spontaneous liquid crystalline organization of the nucleosomes in concentrated solution. The different textures observed in polarizing microscopy suggest the formation of a discotic columnar hexagonal phase (Bouligand, 1980; Chandrasekhar and Ranganath, 1990), sketched in Fig. 3. Each column corresponds to a pile of nucleosomes stacked on each other. The columns form a two-dimensional hexagonal lattice, as reflected by the sixfold symmetry of the homeotropic flower-shaped germs. In the homeotropic germs, the columns of nucleosomes are aligned more or less perpendicular to the preparation plane. Conversely, in the birefringent germs, columns of nucleosomes are aligned in the preparation plane and oriented parallel to the germ long axes.

### Lamellar structure of the columnar phase

The hypothesis of a discotic columnar hexagonal liquid crystalline phase was confirmed by freeze-fracture electron microscopy of frozen specimens. High magnification reveals the presence of multiple juxtaposed domains that are difficult to individualize from their surrounding isotropic environment. These domains are highly ordered. Multiple patterns can be observed and attributed to various fracture planes of the structure, among which two main types can be distinguished:

1. Some fracture planes reveal a lamellar structure. Each layer is formed by columns aligned in parallel. The fracture propagates from one layer to the next by forming a step (Fig. 4, *a* and *b*), with the orientation of the columns remaining constant from one layer to the next. The average distance between neighboring columns can be measured, either directly on the micrographs or by optical diffraction on the negatives, as 11.15 ± 1 nm. In addition, a fine transverse striation can be seen along the columns (Fig. 4 *b*, *arrows*). We suspect that this striation is due to the piling of the nucleosome core particles, and the average distance between two neighboring nucleosomes stacked on top of each other, measured as 7.16 ± 0.65 nm, supports this hypothesis. This stacking is not highly regular, which leads us to suspect that the position of the nucleosome inside the

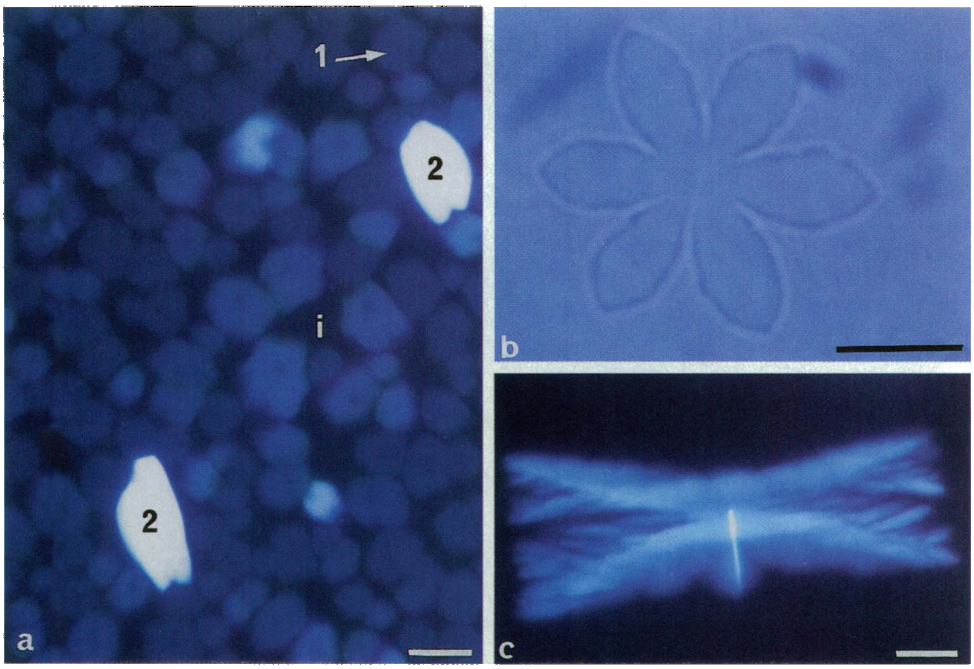


FIGURE 2 Germs of the liquid crystalline phase of nucleosome core particles observed by polarized light microscopy in concentrated solution (scale bars = 20 μm). (*a*) Hexagonal (*1*) and elongated highly birefringent germs (*2*) growing in the isotropic solution (*i*). (P, A 90°). b. Flower-shaped homeotropic germ with a six-fold symmetry (P, A ||). (*c*) Elongated highly birefringent germs with a bilateral symmetry (P, A 90°).

columns is probably not perfectly defined. The distance separating successive nucleosome cores may show fluctuations, which is not surprising in a fluid discotic structure. Moreover, the low resolution in the direction of the columns suggests that the nucleosome cores are free to rotate around their axis, exposing multiple views of their lateral surface to the fracture plane along a given column.

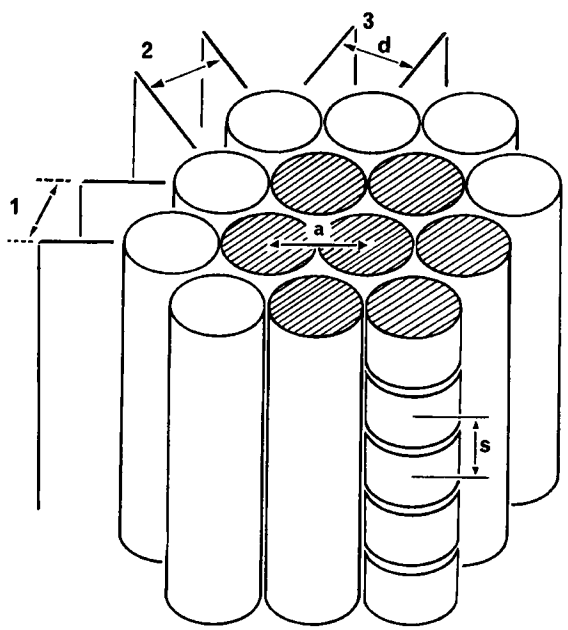


FIGURE 3 Model of the nucleosome core particles arrangement in the liquid crystalline discotic columnar hexagonal phase: the nucleosome cores are stacked on top of each other into columns that form a two-dimensional hexagonal lattice. The three main directions of the network define three series of layers (*1, 2, 3,*) whose thickness (*d*) equals $a\sqrt{3}/2$, where *a* is the distance between two neighbouring columns. In the direction of the columns, *s* corresponds to the average distance separating the nucleosome core particles.

2. Some fracture planes do not allow the visualization of the columns of nucleosomes, but reveal three main directions separated by an angle of 60°. These three directions can be attributed to the three directions of an hexagonal network in transversal section (Fig. 4 *c*). The hexagonal symmetry is confirmed by optical diffraction of the negatives (Fig. 4 *d*). The distance between the reticular planes was measured on diffraction patterns of 26 different liquid crystalline germs. It varies from 8.8 to 11 nm, with an average value of 10 nm. This corresponds to spacings between columns (*a*) varying from 11.16 to 12.7 nm, with an average value of 11.55 nm.

Electron microscopy data confirm polarized light microscopy results and show that in concentrated solution, mononucleosome core particles form a discotic columnar hexagonal liquid crystalline phase.

## Stability of the nucleosome core particles

When the solution is concentrated by the PEG method, the ionic conditions are kept constant in the sample (set at 100 mM and 150 mM NaCl in our experiments). Concentrating the sample by dehydration is accompanied by a progressive increase in the counterion concentration up to 300 mM NaCl. To check the stability of the nucleosome core particles under these experimental conditions (100–300 mM NaCl), an aliquot of an ordered preparation was set apart from a birefringent domain between slide and coverslip, diluted in adequate buffer, and allowed to migrate in acrylamide gel. We did not notice any change in the electrophoretic properties of the nucleosome cores. They remained stable for months over the whole range of nucleosome and

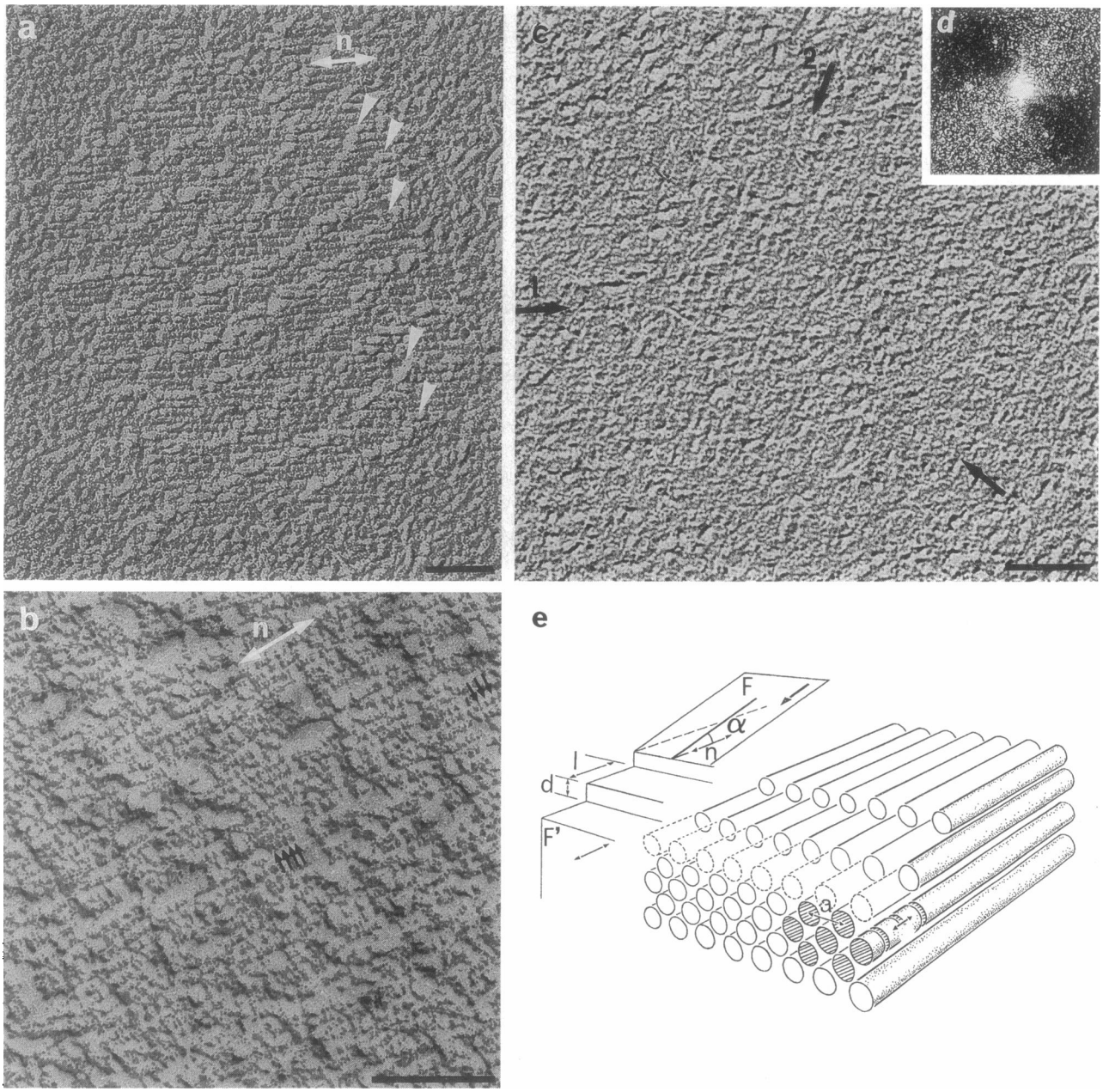


FIGURE 4 Freeze-fracture electron microscopy of concentrated solutions of nucleosome core particles (scale bars = 100 nm). Ordered domains are observed within an isotropic environment. Different patterns can be distinguished: (*a*) Columns of nucleosomes are aligned in parallel along a direction (*n*). Superimposed successive layers of columns are revealed by the fracture process, which forms steps (*white arrowheads*) from one layer to the next. (*b*) At high magnification, a thin striation due to the stacking of the nucleosome cores is visible along the columns (*black arrows*). (*c*) Fracture plane nearly perpendicular to the columns of nucleosomes. The three main directions of the hexagonal lattice are visualized and indicated by arrows. Two directions of the lattice (*1, 2*), underlined by the unidirectional shadowing of the replica, are more easily visible than the third. (*d*) Diffraction pattern of micrograph *c*. (*e*) Interpretative drawing of the freeze-fracture patterns. In most cases, the fracture plane (*F*) forms an angle $\alpha$ with the direction of the columns (*n*). The fracture plane is thus oblique and goes down from one layer of columns to the next, creating a series of steps whose height *d* equals $a\sqrt{3}/2$ (where *a* is the interhelix distance) and length (*l*) is irregular, but varies on average with the obliquity angle $\alpha$. For simplicity, the steps here follow directions normal to (*n*), but this is not a rule, as seen in *a*. The three directions of the hexagonal lattice (*1, 2, 3*) are observed when the fracture plane is normal to *n* (*F'*), as seen in *c*.

NaCl concentrations considered in our study. Moreover, any denaturation of the particles, leading to a separation of DNA from the histone core, could be detected immediately by polarizing microscopy, because DNA segregates from proteins and forms other types of liquid crystalline phases (cholesteric or columnar hexagonal), the textures of which are well known, completely different, and easily recognizable (Livolant, 1986; Livolant and Bouligand, 1986; Livolant et al., 1989). Such effects were never unintentionally created in our preparations.

## Mesophases versus true 2D and 3D crystals

True crystals were obtained previously by different methods of preparation, which include the use of alcoholic solutions and involve the presence of polycations (spermidine or spermine). These methods led to the aggregation of charged particles, which may sediment from the dilute solution and be deposited on microscope grids for EM observations or be handled for x-ray diffraction analyses. In these crystals, the positioning of the nucleosome particles is quite regular, although flexible, thus leading to different structures: 1) hexagonal crystals, in which the nucleosomes form sinusoidal rows by placing the small side of the wedge cylinder (also called a pivot) on opposite sides every three nucleosomes (Finch et al., 1977, 1981; Finch and Klug, 1978); 2) arcs (Dubochet and Noll, 1978) where a specific binding occurs with the pivot on the inside of the arc; and 3) nucleosome cylinders, in which the nucleosomes cores stack into helices of different radii and diameters, which determine the wedge angle of the nucleosome core (Dubochet and Noll, 1978).

In our experiments, the concentration of the discotic mesophase is very close to that of the surrounding isotropic solution. Interactions between nucleosome core particles are also of two types: side-by-side and top-to-bottom, but the columns usually remain straight and do not follow helical or sinusoidal paths, as in these true crystals. Although this should be checked by x-ray diffraction analysis, the position of the nucleosomes along a column is apparently not precisely defined, and the nucleosome cores are free to rotate. Nevertheless, in the absence of curvature, other deviations from the discotic ordering are detected, namely twist and splay between columns (in preparation). This twist probably originates from DNA-DNA interactions between the lateral sides of neighboring nucleosomes. Indeed, a left-handed twist is known to spontaneously appear between right-handed DNA helices, leading to the formation of cholesteric mesophases (Livolant and Maestre, 1988).

## Nucleosome core mesophases as possible model systems for studying the packaging of eucaryotic chromatin

DNA liquid crystalline phases, whose nature depends on the DNA concentration, were shown to be relevant models of the organization of the genetic material inside chromatin devoid of nucleosomes, as in virus, bacteria, lower eucaryotes (dinoflagellates), and certain sperm nuclei (Livolant, 1991). The liquid crystalline phases of nucleosome core particles may be a good model system for studying the organization of the eucaryotic chromatin. Indeed, the nucleosome core concentration in the liquid crystalline phase can be evaluated from the parameter $a$ of the network (11.55 ± 1 nm) and the nucleosome repeat along the column $s$ (7.16 ± 0.65 nm). The concentration $C$ is defined as

$$C = m_{nuc}/\sigma s$$

where $m_{nuc}$ is the mass of a nucleosome core, $\sigma$ is the area of the unit cell of the two-dimensional lattice, and $s$ is the axial translation per nucleosome core. Taking the molar mass $M_{nuc}$ as 204 800 g/mol, $C$ is between 310 and 485 mg/ml (average value 410 mg/ml). This range of concentration is that found for condensed forms of eucaryotic chromatin, for example, 433 ± 74 mg/ml in the chromosome of *Euglena sp.* (Bohrmann et al., 1993). Preliminary experiments show that other liquid crystalline phases can be obtained by varying the nucleosome core particle concentration or the ionic conditions of the solution (Leforestier et al., manuscript in preparation).

Nevertheless, two major limitations of this experimental model must be pointed out. The first is the absence of linker DNA between nucleosome core particles. In chromatin, internucleosomal DNA, the length of which varies from ~20 to 100 bp, ensures the continuity of the chromatin filament and is assumed to be a key element in determining its supramolecular organization. For many authors, both the flexibility (or rigidity) of the linker DNA and fluctuations of its length inside a given nuclei would determine the degree of regularity of the condensed filament (Williams et al., 1986; Athey et al., 1990; Woodcock et al., 1993), whereas for others the packing of nucleosomes in the solenoid would be independent of the spacer length (McGhee et al., 1983; Woodcock, 1994). The consequences of the presence of linker DNA on this liquid crystalline organization could be tested on this model by using di-, tri-, or polynucleosomes. The hypothesis that the continuity of the DNA filament would not prevent the liquid crystalline ordering of chromatin is supported by the work of Luzzati and Nicolaieff (1959, 1963). Studying the organization of gels of nucleohistones as a function of water content and ionic strength by small-angle x-ray diffraction techniques, Luzzati and Nicolaieff have indeed reported the existence of multiple liquid crystalline phases; however, their nature had not been elucidated, at a time when the existence and structure of the nucleosome were still unknown. These experiments should be reproduced under well-defined conditions of preparation, which are now available.

The second major limitation is the absence of H1 histones, which play an essential role in vivo both at the level of the nucleosome particle itself by interacting with the entry and exit DNA segments and in the formation of the higher order structures of the chromatin fiber (Zlatanova, 1990). Preliminary results (Leforestier and Livolant, manuscript in preparation) show that these basic proteins may induce the aggregation of the nucleosome core particles in the absence of any crowding conditions. However, the formation of these aggregates, which separate from the dilute solution, is due to the presence of attractive interactions, contrary to what happens in crowded solutions. A lot remains to be done to understand how these different mechanisms act together to lead to the formation of the higher order organization of chromatin in vitro and in vivo. We therefore intend to take the simple model system presented in this article further, to understand how the condensing

properties of H1 histones (or protamines) may modulate the discotic organization of this mesophase.

A liquid model of chromatin in the eukaryotic nucleus, based on cryoelectron microscopy of intact cells has been proposed by McDowall et al. (1986). We propose that chromatin may not be a simple liquid, but may be locally arranged in liquid crystalline domains, the nature of which remains to be determined. The interest of such a liquid crystalline organization of chromatin in vivo would be its ability to show fluctuations in response to small changes in the physicochemical conditions, by allowing reversible phase transitions to assume changes in the organization of chromatin at different steps of the cell cycle.

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