Large-scale isolation of a native deoxyribonucleohistone complex from baker's yeast

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ABSTRACT

A method for large scale isolation of a native deoxyribonucleohistone complex from yeast is described. Crude chromatin, obtained after disrupting yeast cells at low ionic strength, contains a large amount of lipids, partially due to contaminating membranes. Most of them are removed by a Triton X-100 treatment, followed by step-gradient centrifugation. About 90% of the pellet may be solubilized by mild procedures, the composition of the soluble material being: histone/DNA = 1.0; non-histone proteins/DNA = 0.55; RNA/DNA = 0.18. Histones can be obtained with high purity. Micrococcal nuclease digests DNA to yield a series of oligomeric fragments, with an average repeat length of about 160 base pairs. Circular dichroism spectra show that $[\theta]_{270}$ is reduced by about 30% when compared to pure DNA and that chromosomal proteins are not denatured. These results indicate that the components of the complex conserve the native state.

INTRODUCTION

The structure of chromatin has for years been one of the most troublesome subjects in Molecular Biology. The discovery of the repeating substructure of chromatin (1-3) and the characterization of the basic subunit or nucleosome (4-6) allowed to gain a new insight into the problem of the relationships between structure and function in the eukaryotic genetic apparatus.

The genetic complexity of eukaryotes is, however, leading to an increasing interest in primitive unicellular nucleated organisms, such as *Saccharomyces cerevisiae*. Yeast chromatin is also organized as a repeating array of nucleosomes. Lohr and Van Holde (7) first reported that nuclease digestion of baker's yeast chromatin yields an oligomeric series of DNA sizes, although the repeat length of these fragments is smaller than

that found in higher eukaryotes. Similar results have recently been reported by other workers (8,9) and a careful analysis carried out by Lohr *et al.* (10) has led to the conclusion that at least 80-90% of the yeast genome is involved in a repeating structure, with an average repeat length of about 160 base pairs. These results have been obtained by digesting yeast nuclei (7-10) and, in some instances, yeast chromatin obtained by lysing purified nuclei (7). The preparation of yeast chromatin from isolated nuclei was first reported by Wintersberger *et al.* (11). This method seems to be suitable for the isolation of small amounts of chromatin preparations, whose repeating organization is preserved (7). This procedure cannot be easily scaled up to obtain large amounts of chromatin, because in order to obtain a good yield of pure nuclei, yeast cells have to be previously converted to spheroplasts (11) and this last step is the limiting one when a large mass of cells is to be handled. This virtually precludes undertaking some structural studies that would require larger amounts of a native material.

On the other hand, Tonino and Rozijn (12,13) described a method to isolate a deoxyribonucleoprotein complex directly from yeast cells, without the previous conversion to spheroplasts. This method proved to be adequate to the obtention of histones (13,14), but it involves a vigorous shearing of crude chromatin at low ionic strength, a condition which is known to result in a redistribution of histones, with the subsequent loss of the native state of chromatin (15).

This paper describes a procedure for the large-scale isolation of a native deoxyribonucleohistone complex from baker's yeast, which may be used to carry out a number of structural studies that require a large amount of native preparations.

MATERIALS AND METHODS

Materials

Commercial pressed baker's yeast "Hércules" (Compañía de Industrias Agrícolas, S.A., Valladolid, Spain) was used in this research. Staphylococcal nuclease (E.C. 3.1.4.7) was obtained from Worthington Biochemicals. Phenylmethylsulphonyl fluoride (PMSF) and ethidium bromide were purchased from Sigma Chemical

Co. "Stains all" was from Serva Feinbiochemica and all other reagents were analytical grade.

Isolation of yeast deoxyribonucleohistone

Crude chromatin was obtained by a method based in that of Tonino and Rozijn (13). Yeast was homogenized in 2 vol of a medium consisting of 1 mM MgSO_4 , 5 mM phosphate buffer, pH 6.5, 0.2% PMSF, 1% isopropanol. After centrifuging and collecting the upper brown layer of the sediment as described by Tonino and Rozijn (13), the collected sediments were washed twice with the same medium by gently suspending and centrifuging for 40 min at 8,000 g. The sediment was washed with 0.14 M NaCl, 5 mM tris-HCl, pH 7.5 (20 min, 8,000 g) until the supernatant became clear. Five or six washings with 6 vol of buffered saline were usually necessary. The remaining sediment was gently suspended in 0.25 M sucrose, 1 mM MgSO_4 , 0.5% Triton X-100, 5 mM tris-HCl buffer, pH 8.0 and centrifuged for 15 min at 8,000 g. The sediment was washed twice with the Triton-containing medium, once with 1 mM MgSO_4 , 5 mM tris-HCl buffer, pH 8.0 (10 min at 15,000 g) and it was resuspended in the latter buffer to a concentration of about 0.5 mg DNA/ml. The suspension, in aliquots of 10 ml, was layered on the top of a sucrose gradient consisting of 15 ml 2.0 M sucrose and 15 ml 1.5 M sucrose, both in 1 mM MgSO_4 , 5 mM tris-HCl buffer, pH 8.0 (13) and centrifuged in a fixed-angle rotor at 35,000 g for 2 h. The pellet was recovered; it will be further referred to as pure deoxyribonucleohistone.

Extraction of histones

Pure deoxyribonucleohistone was washed twice with 0.14 M NaCl, 5 mM tris-HCl, pH 7.5 (8,000 g, 20 min) and extracted with 0.25 N HCl as previously described (14).

Nuclease digestion

The pellet of pure deoxyribonucleohistone was washed with digestion buffer (16) and digested with micrococcal nuclease as described by Woodhead and Johns (17). Reactions were carried out for several time periods, from 3 to 30 min. DNA fragments were extracted following the procedure of Britten *et al.* (18).

Circular dichroism spectra of deoxyribonucleohistone

The pellet of pure deoxyribonucleohistone was washed once with 10 vol of distilled water (18,000 g, 10 min). The pellet was gently resuspended in 10 vol of 5 mM tris-HCl buffer, 1 mM sodium ethylenediaminetetraacetate, pH 8.0 and dialyzed against 25 vol of the same buffer (five changes). This treatment resulted in the solubilization of 90% of deoxyribonucleohistone (see below). Soluble deoxyribonucleohistone was recovered in the supernatant, after centrifuging at 35,000 g for 30 min.

Circular dichroism (CD) spectra of soluble deoxyribonucleohistone were recorded in a Mark III dicrograph (Jobin-Yvon). For routine measurements, spectra were obtained after diluting the samples with the solubilization buffer to $A_{260} \approx 1.5$. A cell with 1 cm optical path was used in the region from 255 to 330 nm, whereas a 0.5 mm cell was utilized from 200 to 255 nm. The instrument was adjusted to a spectral band width of 0.2 nm for wavelengths higher than 250 nm. For measurements at lower wavelengths, the spectral band width was stepwise increased up to 2 nm. Ellipticities were reported as molar ellipticities, with the dimensions of $\text{deg}\cdot\text{cm}^2/\text{dmole}$, based on the concentration of DNA phosphorus over 250 nm and on protein residue concentration below this wavelength.

Determination of the chemical composition of deoxyribonucleohistone

Chemical composition of deoxyribonucleohistone was determined by a method based on that of Hill *et al.* (19). Deoxyribonucleohistone was extracted overnight with 0.25 N HCl with constant shaking at 2°C, and centrifuged (17,000 g, 20 min). The sediment was re-extracted for 3 h with 0.25 N HCl. The combined supernatants were made 20% in trichloroacetic acid and the precipitated histones were collected by centrifuging for 20 min at 20,000 g and the sediment was redissolved in 0.1 N NaOH. Lipids were extracted from the sediment remaining after the acid extraction by the procedure of Bligh and Dyer (20). Lipids were determined in the organic phase, and both the upper phase and the material precipitated at the interphase were carefully removed and centrifuged at 4,000 g for 5 min. The sediment was hydrolyzed in 0.3 N KOH at 37°C for 3 h. The reaction was stop-

ped by adding 0.05 vol of ice-cold 60% perchloric acid. The sediment was washed once with 0.25 N perchloric acid, and the combined supernatants were analysed for RNA. The washed sediment was hydrolyzed in 0.15 N perchloric acid (95°C, 15 min), the reaction being stopped and the sediment washed as before. The combined supernatants were saved for DNA analysis and the remaining sediment was dissolved in 0.1 N NaOH for non-histone protein determination.

Proteins were determined by the method of Lowry *et al.* (21). DNA and RNA were determined by the procedure of Webb and Levy (22). Total lipids were transferred to preweighed vials and determined by gravimetry. Total lipids were fractionated into the different lipid classes by thin-layer chromatography on silica-gel G (23). Polar lipids were fractionated by two-dimensional thin layer chromatography (24).

Other analytical methods

Electrophoresis of histones was carried out in 15% polyacrylamide, 2.5 M urea gels (25). Gels were stained with amido black and scanned in a Canalco Model G densitometer, with a 570 nm Wratten filter. DNA fragments were analysed by the method of Loening (26). Gels were stained with either ethidium bromide or "Stains all". In the latter instance, they were scanned as above.

RESULTS

Isolation and composition of yeast deoxyribonucleohistone

The chemical composition of yeast deoxyribonucleohistone at the main stages in the purification procedure is given in table I. The high lipid/DNA ratio in crude chromatin is particularly noteworthy. This ratio was largely dependent on the yeast batch used for the extraction, but it always was higher than 30 (see table I).

The presence of lipids in some chromatin preparations has been described by several authors (27,28) but this fact usually reflects contamination by membrane materials. In the present case, lipids seemed unlikely to proceed entirely from membranes associated to chromatin. First of all, the lipid/DNA ratio in

TABLE I

CHEMICAL COMPOSITION OF YEAST DEOXYRIBONUCLEOHISTONE

Stage of DNH [†] preparation	Yield ¹	Protein ²		RNA ²	Lipids ²
		Acid-soluble	Acid-insoluble		
Crude DNH	100	2.0	8.0	1.0	30-50*
Triton-washed DNH	80	1.3	5.2	0.6	1.6
Pure DNH (pellet)	35	1.1	3.5	0.3	
Soluble DNH	32	1.0	0.55	0.18	0.7

Results given in this table are the average of four experiments. Except for the values marked with an asterisk, they were reproducible within an error interval of $\pm 0.5\%$.

[†] DNH = Deoxyribonucleohistone.

¹ Expressed as the percentage of DNA recovery.

² Expressed as mg per mg DNA.

crude chromatin is too large to be accounted for the presence of membranes. On the other hand, if the presence of lipids were only due to the association of membranes, a higher level of proteins would be expected in crude chromatin, since proteins roughly account for 50% of the yeast membranes (29). An excess of protein is actually present in yeast deoxyribonucleoprotein at this stage of the preparation (table I), but its amount ought to be much higher if a protein counterpart would correspond to all the isolated lipids.

Lipids from crude chromatin were analysed as described under Material and Methods. Neutral lipids were more abundant than the polar ones. The major lipid classes were sterol esters, phosphatidylethanolamine, triacylglycerols, free fatty acids, phosphatidylcholine, free sterols and diacylglycerols. Some other minor classes and an unidentified lipid, with a relative abundance similar to that of phosphatidylethanolamine, were also found. The unidentified lipid was somewhat polar and it showed a high degree of insaturation.

Most of the lipid classes found in crude chromatin have been reported to be present in yeast membranes (29), but the most prominent one, *i.e.* sterol esters, is unlikely to proceed

from membranes. It is, thus, conceivable that the presence of lipids in yeast crude chromatin may be in part due to contaminating membranes, while the rest of the lipids may have been trapped within chromatin fibres. As a result of the presence of lipids, the density of crude chromatin is low and it does not enter the 2.0 M layer of the sucrose gradient.

In order to obtain a pure and soluble deoxyribonucleohistone preparation, membranes and lipids from other sources have to be removed. Navlet (30) found that shearing of crude chromatin at low ionic strength, as described by Tonino and Rozijn (13), resulted in the removal of lipids, and the deoxyribonucleoprotein complex obtained could be then sedimented in 2 M sucrose. As pointed out before, shearing at low ionic strength may disrupt nucleosomal structure (15), and in order to minimize this risk, we tried to remove the lipids following the procedure of Tonino and Rozijn (13), except that shearing was carried out in the presence of 0.14 M NaCl. According to Woodhead and Johns (17), this treatment preserves the nucleosomal structure of chromatin. However, it did not result in the elimination of lipids from crude chromatin; it was still unable to enter the 2.0 M sucrose layer of the gradient and its chemical composition remained unchanged. Nevertheless, lipids were efficiently removed by treating crude chromatin with non-ionic detergents, such as Triton X-100, as described under Materials and Methods.

Table I shows the effects of Triton X-100 treatment on crude chromatin. About 50% of the Triton-washed material could be sedimented in 2.0 M sucrose. This step and the subsequent solubilization resulted in the removal of most of the RNA and acid-insoluble proteins. Figure 1 shows the electrophoretic patterns of acid-soluble proteins, separated by polyacrylamide gel electrophoresis. The slight loss of acid-soluble proteins during the solubilization step may be related to the disappearance of slow-moving non histone proteins. Nearly all the acid-soluble proteins in soluble deoxyribonucleohistone may be identified as histones. The band marked with an asterisk in figure 1 has the same electrophoretic mobility as calf thymus H1. This material has been previously observed by other workers

Figure 1. Electrophoretic patterns of: (A) yeast histones from soluble deoxyribonucleohistone prepared as described under Materials and Methods; (B) yeast crude histones from the pellet of pure deoxyribonucleohistone; (C) yeast histones prepared by the procedure of Tonino and Rozijn (13). Migration is from left to right in 15% polyacrylamide-2.5 M urea gels (25).

and its nature remained unclear (8,31) until the recent work of Spiker *et al.* (32), who have identified it as an HMG non-histone protein. If the low content of acid-soluble proteins other than histones is taken as a criterion of purity of the deoxyribonucleohistone, the procedure described in this paper gives a final preparation whose purity is comparable with that of the deoxyribonucleohistone obtained by the method of Tonino and Rozijn (13) (see figure 1, A and C). Chemical purity of yeast deoxyribonucleohistone is also evident from the data given in table I. The somewhat high ratio RNA/DNA is consistent with the high transcriptional activity of yeast chromatin. Table I also shows that some residual lipids were still bound to Triton-washed deoxyribonucleohistone. The most prominent lipid class was, in this instance, the above-mentioned unidentified lipid.

Micrococcal nuclease digestion of yeast deoxyribonucleohistone

It appeared from the data stated above that the deoxyribo-

nucleohistone complex obtained by the method described in this paper had a reasonable chemical purity, comparable to that of the complex obtained by Van der Vliet *et al.* (33). In order to check whether the native state had been preserved during the purification procedure, pure deoxyribonucleohistone was digested with micrococcal nuclease as described under Materials and Methods. Figure 2 shows the electrophoretic patterns of DNA fragments from yeast deoxyribonucleohistone and calf thymus chromatin. A logarithmic plot of the band number *versus* mobility gave a straight line for the five major bands in curve A. This fact indicated that nuclease had cut DNA at regular intervals, and that the sizes of slow-moving bands are integral multiples of the size corresponding to band I. Increasing the digestion period to 15 min had the effect of diminishing the in-

Figure 2. Electrophoretic patterns of DNA fragments from micrococcal nuclease digests of: (A) Yeast deoxyribonucleohistone complex, prepared as described under Materials and Methods (10 min digest); (B) Yeast deoxyribonucleohistone (15 min digest); (C) Calf thymus chromatin (10 min digest, prepared according to Woodhead and Johns (17)). Migration is from left to right in 5% polyacrylamide gels (26). Gels were stained with "Stains all" and scanned at 570 nm. Inset: Logarithmic plot of band number *vs.* mobility for the yeast 10 min digest (curve A).

tensity of polymer bands, and the disappearing material accumulated in the monomer band. This kinetic feature led to conclude that the band marked I in the yeast digests originated from the slow-moving bands, this fact supporting their assignment to monomers and oligomers respectively. It is also evident from figure 2 that the mobility of the discrete DNA fragments from yeast deoxyribonucleohistone is higher than that of the fragments from calf thymus chromatin. This result indicates a smaller repeat size in yeast chromatin. After extrapolating to zero time the data shown in figure 2 and those obtained for shorter times, a rough estimation of the monomer size gave a result of about 150-160 base pairs, in close agreement with the values reported by other workers for the nucleosomal size in yeast (7-10). The limit digest size was roughly estimated at 135 base pairs. These facts constitute an additional evidence that the discrete DNA fragments observed in figure 2, A and B actually correspond to nucleosome monomers and oligomers. As far as to the DNA organization is concerned, these data may be taken as a proof of the repeating native state of the deoxyribonucleohistone complex obtained by the method described in this paper.

The three bands that appear ahead of the monomer band in the yeast digests (figure 2) were not detectable in ethidium bromide stained gels and they may correspond to the "mystery bands" described by Van Holde and his colleagues (10).

CD spectrum of yeast deoxyribonucleohistone

In order to obtain a further characterization of the yeast deoxyribonucleohistone complex, some optical properties were investigated. The absorption spectrum (not shown) was characteristic of nucleoproteins and corresponded to that obtained by Van der Vliet *et al.* (33) for sheared soluble deoxyribonucleohistone from yeast. The CD spectrum provided a more valuable information. Figure 3 shows the spectrum of yeast deoxyribonucleohistone obtained as described under Materials and Methods. Its shape shows the characteristic features of a CD spectrum of chromatin, *i.e.*, a maximum at about 270 nm, a shoulder at 280 nm, and a small minimum between 290 and 300 nm (34). The resemblances between the spectrum shown in figure 3 and those of

Figure 3. CD spectrum of yeast soluble deoxyribonucleohistone. See the text for details.

chromatin preparations from higher organisms are also evident from a quantitative point of view. The molar ellipticity at 273 nm is 6,200 $\text{deg}\cdot\text{cm}^2/\text{dmole}$, in close agreement with the values reported for chick embryo brain chromatin, which also has a high RNA content (34).

The examination of the far ultraviolet region of the CD spectrum showed that chromatin proteins had not been substantially denatured during the preparation of the deoxyribonucleohistone complex. Neglecting the minor contribution of nucleic acids to the ellipticity at 222 nm ($-8,200 \text{ deg}\cdot\text{cm}^2/\text{dmole}$), the amount of helical structure in the proteins of the complex may be estimated at about 20%, by using the procedure of Chen and Yang (35).

DISCUSSION

It seems from the evidence given here, that the method described for the isolation of a deoxyribonucleohistone complex from yeast does not alter the native state of the chromosomal components. The patterns of nuclease digestion clearly indicate that the repeating structure of yeast chromatin is not lost

during the isolation procedure. As indicated above, both the size of the repeating fragments and the increase in the concentration of the faster migrating fragments at the expense of the slow-moving ones during the time-course of nuclease digestion allows the identification of the discrete DNA fragments with nucleosomal DNA. Preliminary evidence (López-Braña, unpublished results) also shows that DNAase I makes single-strand cuts at multiples of 10 bases in deoxyribonucleohistone prepared by the Triton X-100 method.

The examination of the CD properties of deoxyribonucleohistone above 250 nm gives information on the protein-induced changes in DNA structure. The ellipticity of the main positive band in the spectrum (figure 3) is reduced by about 30% from that of pure DNA. This depression of the positive DNA band is a distinctive feature of chromatin and deoxyribonucleohistone complexes. Actually, the CD of DNA in chromatin seems to be intermediate between the spectra of B and C forms of DNA (36), but this fact does not imply the real presence of C form, and the reduction in ellipticity may arise from the partial base unstacking associated with the superhelical organization of nucleosomal DNA. The possible causes that affect the CD spectrum of DNA in chromatin have been discussed by Fasman (37). Whatever the causes of the CD changes may be, the finding of such a change in the deoxyribonucleohistone complex may be an indication that the structure of DNA in the complex is similar to that of DNA in native chromatin, at least from a qualitative point of view. Quantitative conclusions cannot, however, be easily drawn, because RNA significantly contributes to the ellipticity of chromatin around 270 nm. In fact, Hjelm and Huang have shown that the greater the content of RNA in chromatin, the higher the ellipticity at 273 nm (34). This fact may explain that, in the yeast deoxyribonucleohistone complex, the ratio $[\theta]_{273}/[\theta]_{283}$, which has been estimated at 1.25 from the spectrum shown in figure 3, is higher than those found in several mammalian chromatins, whose RNA content is smaller (34).

Strictly speaking, the evidence obtained from the CD data do not prove that the yeast deoxyribonucleohistone complex is native, but that DNA has a structure whose optical properties,

and hence its structure as a whole, resemble those of DNA in native chromatin. When this fact is considered together with the evidence obtained from nuclease digestions, it may be concluded that the main fine details of the repeating structure of chromatin, and not only the average structure of DNA, have been preserved during the extraction procedure.

As far as to the chromatin proteins is concerned, the CD spectrum may indicate a substantial conservation of their secondary structure. Our estimate of 20% helical structure in the proteins of the complex is in good agreement with the values found by Hjelm and Huang (36) in native chromatin preparations.

Thus, the yeast deoxyribonucleohistone complex isolated by the Triton X-100 method may be regarded as native by a number of criteria, which affect both DNA and protein structures. The question arise as to whether this complex may be considered as native chromatin. Failing a definite experimental criterion to define the chromatin, it would be desirable to proceed very cautiously when referring to some nucleoprotein complexes as chromatin. This reason leads us to avoid the term chromatin in the designation of the deoxyribonucleohistone complex. Actually, although there is clear evidence that the native state of its components is preserved, the assumption that no component of native chromatin is missing would be rather questionable. For instance, Johns and his colleagues have found that some HMG proteins may be removed from chromatin by Triton X-100 treatment (Johns, E.W., personal communication). In the present case, however, assuming that the band marked with an asterisk in figure 1 corresponds with the yeast HMG reported by Spiker *et al.* (32), one might conclude that some HMG protein is present in the Triton-washed yeast deoxyribonucleohistone complex.

Anyhow, the procedure described in this paper yields a deoxyribonucleohistone preparation which may be useful for a number of purposes. First of all, it may be used as starting material for the large-scale isolation and fractionation of yeast pure histones (Belmonte *et al.*, manuscript in preparation). On the other hand, a number of structural studies that would require large amounts of native chromatin may be readily carried out with our deoxyribonucleohistone complex, and the

possibility of obtaining large amounts of nucleosomal core particles should be kept in mind. Moreover, the absence of an H1 histone similar to that of higher organisms in yeast chromatin (8,14,31) makes possible the development of research on the role of this histone, by using the yeast deoxyribonucleohistone complex; this work is now in progress.

Finally, we wish to comment on the presence of residual lipids in the deoxyribonucleohistone complex. Although we do not know whether these lipids have to be actually considered as chromatin components or not, it has to be noted that Fazal and Cole (38) have found that lipids are co-extracted with wheat germ histones and Mardian and Isenberg have recently suggested that yeast histones may also bind lipids, or other yeast constituents, which alter their properties (39). This assumption may, of course, be in connection with the presence of residual lipids in the deoxyribonucleohistone complex.

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