Ultrastructure of transcriptionally competent chromatin

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ABSTRACT

We have examined a salt-soluble, transcriptionally competent gene-enriched fraction of chicken erythrocyte chromatin and compared it to bulk chromatin using the unique microanalytical capabilities of Electron Spectroscopic Imaging (ESI). The salt-soluble fraction is enriched 48 fold in β -globin gene sequences and is also enriched in histones that are post-synthetically modified, including acetylation and ubiquitination. Differences between the two fractions are also apparent in the distribution of the two major forms of nucleoprotein structures, including (1) particles which present a circular profile and possess protein and DNA content nearly identical to that of the canonical nucleosome and account for 89% of particles in the bulk fraction but account for only 66% of the particles in the competent fraction, and (2) u-shaped particles which possess about 20% less protein mass than particles of circular profile and are about 10x more prevalent in the transcriptionally competent fraction than in the bulk. Additionally, elongated particles with protein and DNA content similar to the u-shaped objects are also seen in the competent fraction.

INTRODUCTION

The canonical nucleosome core particle is characterized as a wedge-shaped disk, 11.0 nm in diameter and 5.7 nm in height. It comprises a histone octamer core containing two each of histones H2A, H2B, H3 and H4, around which is wrapped approximately 1.8 negatively supercoiled turns of DNA corresponding to 146 base pairs (1,2). Structural information on the most fundamental repeating unit of chromatin is the starting point for understanding the role of chromatin structure in gene regulation. A number of biochemical and biophysical studies have been undertaken to distinguish between nucleosomes in transcribing and inactive chromatin, from which several models for the transcriptionally permissive nucleosome have arisen(3,4,5). Active genes exhibit an altered sensitivity to nucleases such as MNase I and DNase I and II compared to

inactive genes(2). Despite many attempts, the structure of the transcriptionally permissive nucleosome is still not well-defined, possibly as a consequence of the limitations of the experimental techniques employed. For example, nuclease digestion studies do not clearly reveal the structural basis of the difference between active and inactive chromatin 'cutting patterns', and fine structural detail is partially obliterated in conventional electron microscopy by staining and shadowing techniques. Moreover, information pertaining to DNA and protein content has had to be generated from separate analytical assays. Both scanning transmission electron microscopy (STEM) (16) and Electron Spectroscopic Imaging (ESI), an electron microscopic technique, can partially overcome these limitations. To supplement the body of structural and biochemical information we have begun to apply ESI to the study of transcriptionally competent and inactive chromatin.

Electron microscopy can be used to visualize large domains of chromatin at both low and intermediate resolution and provides the ability to visualize structurally unique populations in an ensemble of complexes. The distinguishing feature of ESI is its additional capability to detect quantitatively, and to map the distribution of a specific chemical element within a thin section or macromolecular spread(6). Phosphorus distributions in nucleoprotein complexes can be used to distinguish between the protein and nucleic acid moieties since phosphorus is a major component of nucleic acid but is present at much lower concentrations in protein. ESI's utility in imaging DNA has been well established(7) and ESI has already been used to examine a variety of nucleoprotein structures including nucleosomes(8,9), RNA-protein complexes(7), TFIID-5S DNA complexes(6) and ribosomal subunits(10). In this paper we show that the majority of chromatin subunits from salt-soluble, transcriptionally competent gene-enriched and bulk inactive chromatin do not, on average, differ significantly in DNA or protein content. Nevertheless, the subunits in the samples of chromatin are not monomorphic but exhibit heterogeneity with respect to DNA and protein content. Furthermore, on the basis of ultrastructural classification, several distinct groups become apparent. One of these groups appears to be enriched within salt-soluble, transcriptionally competent gene-enriched chromatin and may represent a transcriptionally permissive form of the nucleosome.

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MATERIALS AND METHODS

Isolation and Fractionation of Chromatin

Bulk chromatin (fraction SEDTA), salt-soluble chromatin (150 mM NaCl-soluble chromatin) or salt-soluble polynucleosomes (Bio-Gel A-5m column fraction F1) were prepared by the method described by Ridsdale and Davie(11,12). The fractionated material (approximately 3 A260 units/ml in 10 mM EDTA, pH 7.5) was fixed in 1% formaldehyde for 1 minute at 0°C, diluted to 0.1% in 10 mM Tris pH 8.5, 1 mM EDTA and dialysed against Tris-EDTA at 4°C. Chicken erythrocyte mononucleosomes were generously provided by Dr. Rafael Oliva, University of Calgary, prepared as described (13). The content of modified histone forms in the chromatin fraction (fraction SEDTA and column fraction F1) was determined by electrophoretically resolving the modified histone species on acetic acid/urea/Triton X-100 15% polyacrylamide gels, and scanning the Coomassie Blue-stained gels with a densitometer (11).

Preparation of Specimen Grids

Formaldehyde fixed chromatin samples were diluted to approximately 30 µg/ml in 10 mM Tris, 2 mM MgCl₂. A 5µl volume was applied to a 1000 mesh EM grid previously covered with a 2 to 3 nm thick carbon film. After one minute the grid was washed in distilled water, and was allowed to air dry after all but a thin layer of water had been blotted off. Some grids were rotary shadowed at 45° with Pt-Pd (80:20).

Electron Spectroscopic Imaging, Data Acquisition and Analysis

ESI was performed using a Zeiss EM902 electron microscope equipped with a prism-mirror-prism type electron imaging spectrometer. The instrument was operated at an accelerating voltage of 80 kV. An energy selecting slit aperture corresponding to 18 eV, an 800µm condenser aperture and a 60µm objective aperture were used. To produce a map of the phosphorus distribution a reference image taken at an energy level below the phosphorus L_{2,3} ionization edge and a phosphorus enhanced image taken on the maximum of the ionization spectral peak are required. Thus micrographs were recorded using electrons which had lost 120 eV or 160 eV, corresponding to 'reference' and 'phosphorus-enhanced' images, respectively. To generate the 120 eV and 160 eV images, the specimen was exposed to 0.6×10^5 and 1.1×10^5 electrons/nm², respectively.

Images were recorded on Kodak SO-163 electron image film to an average optical density of approximately 0.4 and were processed in full strength Kodak D-19 developer for 12 minutes. Areas of interest on the micrographs were digitized using a linear, high resolution video camera (Bosch) connected to an image analysis computer (IBAS/Kontron). To compensate for differences in photographic intensity between corresponding images, normalization factors were calculated over large regions in the reference and phosphorus enhanced images which were assumed to be devoid of phosphorus containing structures. After normalization, subtraction of the reference image from the phosphorus-enhanced image produces a net phosphorus signal with both high sensitivity and high spatial resolution. A two parameter fit using two reference images, recorded at 100 eV and 120 eV, gave similar results when the relationship of intensity (I) of the spectrum and energy loss (ΔE), $I = A \Delta E^{-R}$, was used(14), where A and R represent constants which must be calculated for each point in the image. To calculate DNA content,

differences in grey value between the reference and phosphorus enhanced images integrated over the molecule were compared to the integrated differences over an internal standard containing a known amount of phosphorus. Because optical density in the reference image is proportional to mass, total mass of a particle could be obtained using integrated optical densities after subtraction of the average optical density contribution by the carbon support film, calculated over a large area around the particle. Glutamine synthetase and exogenously applied chicken erythrocyte chromatin mononucleosomes were used to check the validity of using internucleosomal linker DNA as an internal mass and phosphorus standard.

Individual particles were delimited by an interactive software program on the image analysis computer (IBAS, Kontron) that would generate boundaries at a chosen optical density just above the background optical density. Subunits were included in the analysis regardless of morphologic characteristics provided that they could be resolved as discrete entities and were connected by DNA fibres to adjacent subunits. Objects of nucleosome size which contained no detectable DNA were not included in the analysis. These were observed at a frequency of about 1% in both bulk and salt-soluble chromatin. Large aggregates of subunit particles were also avoided. Sufficient numbers of individually spaced particles were present in both bulk and salt-soluble fractions, but for about 15% of the particles, boundary determination became subjective where two chromatin subunits were in close proximity because the computer would link these objects. In these circumstances a corrected boundary between each subunit was drawn by hand using a mouse. Variation in repeated integrated optical density measurements of the same objects was almost always within 10% of the mean values. Boundary delineation was the major source of experimental error in the mass measurements, but was significantly reduced by taking the average of two or three measurements of each particle.

Statistical analysis of the data was performed on the Biomedical Data Processing Package 82.0 (BMDP, Copyright University of California) using t-tests in program P3D. All statistical data is shown as mean $\pm$ standard error of the mean.

RESULTS

ESI Images of Chromatin

We used ESI to compare the structure of bulk chromatin and a salt-soluble, transcriptionally competent gene-enriched fraction of chicken erythrocyte chromatin. The salt-soluble, transcriptionally competent gene-enriched chromatin (150 mM NaCl-soluble chromatin or salt-soluble, Bio-Gel A-5m fraction F1 polynucleosomes) are enriched in transcriptionally competent DNA, including β -globin, ϵ -globin, histone H2A.F and histone H5 DNA sequences (11,12). Furthermore, salt-solubility properties of the chromatin of various genes correlate with their DNAase sensitivity (12). Transcriptionally repressed DNA sequences (eg., vitellogenin and ovalbumin) are found primarily in the salt-soluble mononucleosomes (11,12). These results suggest that the majority of the salt-soluble polynucleosomes, which represent approximately 1% of the nuclear DNA, are from DNAase I sensitive, transcriptionally competent domains of the genome. The salt-soluble fraction differed from bulk chromatin with respect to content of post-synthetically modified histones and linker histone content with the salt-soluble polynucleosomes being enriched in acetylated and ubiquitinated histones and depleted (0.7-fold) in histones H1/H5 (11,12) (Table I). Bulk

chicken erythrocyte chromatin tended to aggregate into clumps on EM specimen grids with only a relatively small number of 10 nm fibres extending out from the aggregates (Figure 1, upper row). In contrast, salt-soluble chromatin tended to form more extended oligomeric fibres where the majority of subunits were visible as discrete entities (Figure 1, lower row). This difference probably reflects the higher level of histone H1/H5 present in the bulk fraction and is consistent with previous studies which indicate that chromatin containing H1 cannot be spread easily for high resolution microscopy(15).

A comparison can be made of the resolution and clarity of oligomeric nucleosomal chromatin from the salt-soluble fraction imaged by ESI or conventional electron microscopy. An electron spectroscopic inelastic dark field image (right) and a conventional bright field image (left) are shown in Figure 2. Because of the high contrast in the ESI dark-field mode, the chromatin, supported on very thin carbon films, can be imaged without heavy metal stain or shadow, whereas platinum-palladium is used to provide contrast in the bright field image. Both imaging modalities exhibit the typical beads-on-a-string pattern of nucleosomes. In the conventional micrograph, however, the ability to observe

heterogeneity with respect to size and shape of the particles is diminished by the clustering of heavy-metal atoms that comprise the contrast agents. Moreover, heterogeneity in protein and DNA content cannot be addressed using conventional transmission electron microscopy (TEM). Alternatively, the Scanning Transmission Electron Microscope (STEM) has been used to document structures present in chromatin that are often not visible by conventional TEM(16).

Morphological Features of Salt-Soluble Chromatin

Electron spectroscopic images of chemically fixed salt-soluble chromatin reveal two major classes of subunits, particles with a circular profile and with reduced mass in their centres, and u-shaped particles. These two classes comprise 66% and 22%, respectively, of the subunits visualized in this fraction. In addition, approximately 8% of the subunits have an elongated appearance. These three structural classes are represented in Figure 3, where the long arrows indicate objects of circular profile, the short arrows indicate u-shaped subunits, and the arrowhead in (f) indicates an elongated structure. In some cases, insufficient contrast prevents an unambiguous designation of a complex to one of these three categories. Overall, however, the subjective limitation in categorizing the particles is not a major problem. A collection of these three classes of objects are shown in Figure 4. Rows (a) and (b) include objects of circular profile and reduced mass density in the centres, row (c) includes u-shaped objects, and in row (d) an arrangement from left to right of u-shaped to elongated structures illustrates that the elongated objects may be elongated u-shaped objects. This will be discussed later when the protein and DNA content of these structures is presented.

The chromatin in the fixed salt-soluble chromatin fraction appears to have an irregular linker length, in agreement with previous TEM examination of bulk and salt-soluble fractions from chicken erythrocyte chromatin(12). Variable linker lengths can be observed in Figures 2 and 5. Some nucleosomes actually contact each other, whereas others are separated by a linker as long as 100 bp. A possible explanation for the long linkers and, therefore, the variation in linker lengths is that nucleosomes

Table I. Comparison of 150 mM salt soluble polynucleosomes with bulk chromatin

Feature	Bulk % of total histone class	Salt-soluble
<i>Histone H4</i>		
Unacetylated	49	29
Monoacetylated	37	29
Diacetylated	10	21
Triacetylated	3	13
Tetraacetylated	1	8
<i>Histone H2B</i>		
Unacetylated	52	35
Monoacetylated	45	32
Diacetylated	0	12
Triacetylated	0	9
Tetraacetylated	0	3
Utiqitinated	3	9

Figure 1. Inelastic spectroscopic dark field images, recorded at an energy loss of 120 eV, of (a) bulk chromatin (upper row) and chromatin from the salt-soluble fraction (lower row) isolated from chicken erythrocyte nuclei. The chromatin is spread on a carbon film of 2–3 nm thickness. Scale bar represents 54 nm.

become disrupted and fragmented due to the spreading forces when the chromatin attaches to the EM support films. Although this explanation is unlikely because the material has previously been chemically fixed, it can be tested by ESI. In conventional microscopy, fragments of nucleosomes would be obscured by the staining and shadowing compounds. With ESI, however, small amounts of protein on the linker DNA can be detected by direct visualization. In addition, linkers that are composed of DNA and contaminating protein will underestimate the mass of nucleosomes when used as internal mass standards. To test this, the mass of some of the subunits shown in Figure 5 have been measured. The mass measurements are based on the assumption that the linkers between nucleosomes numbered 2 and 3, and 3 and 4 are naked DNA. These two linkers predicted mass values within 10% of each other for all nucleosome subunits, 1–3 and 5–8. The linker between 2 and 3 predicts the mass of these subunits to be 183, 229, 199, and 203, 214, 240, 189 kD, respectively. These linkers do not underestimate the values for the mass of the nucleosomes, based on the canonical molecular weight. Nucleosomes adjacent to a longer linker (between nucleosome 2 and 3) are of equivalent mass to those connected by shorter linkers (nucleosomes 5–8) where the nucleosomes are in close contact with each other. An exception is observed in this field. The object labelled 4, has a mass of 110 kD, just over 50% of the mass of a canonical core particle. This object is an example of the association of a long linker with an object of subnucleosomal mass. That many complexes of canonical nucleosome mass, however, are connected by long linkers supports the interpretation that the long linkers are not arising from disrupted nucleosome subunits. This result is consistent with recent evidence from a variety of genes where nonuniformity in linker length results from DNA sequence preference of histone octamers(17,18).

Mass and DNA Content of Nucleosomal Chromatin

Linker DNA was used as an internal standard from which the mass and phosphorus content of the chromatin subunits in salt-soluble and control chromatin fractions could be calculated.

Figure 2. Images of chromatin from the salt-soluble chromatin fraction from chicken erythrocyte nuclei. The image on the left is a conventional bright field image of a specimen that was rotary shadowed with Pt-Pd. At the right is a phosphorus enhanced inelastic dark field image recorded at an energy loss of 160 eV. Scale bar represents 38 nm.

Linker strands used to make these measurements had to meet the criteria of being uniform in width, 2–3 nm, of constant axial density and of exhibiting an increase in contrast in the phosphorus enhanced image. Two or three linker regions were generally represented in any one digitized field, permitting the comparison of predicted molecular weights of the chromatin subunits using each linker strand. Occasionally, a particular linker would underestimate the molecular weights of subunits by as much as 30%, compared to the values predicted by other linker strands in the field. This was likely a result of protein association with the linker DNA that did not substantially change the apparent diameter of the fibre. In such cases, these values were not used. Only mass values were considered when the values predicted by two or more linkers agreed to within 10% of each other. The ability to use DNA strands to predict molecular mass has already been verified(6), and the use of plasmid DNA as a mass standard cospread with glutamine synthetase predicts the molecular weight of the protein to within 10% on average, values ranging from 6–15% (unpublished data). To further confirm the efficacy of linker DNA as an internal standard, some samples of chromatin were cospread with chicken erythrocyte nucleosome core particles, prepared and purified separately. These were appropriate standards because their mass and DNA content were similar or identical to the complexes being characterized. Protein content of the purified core particles was determined by SDS-polyacrylamide gel electrophoresis, and DNA content was assayed by agarose gel electrophoresis. Each indicated that these were canonical core particles containing equal molar ratios of the four core histones and 146 bp DNA(13). As a final verification, the mass and phosphorus content of purified chicken erythrocyte nucleosome core particles were measured using free DNA as a molecular weight and phosphorus standard (histograms, Figure 6).

The total mass of the canonical core nucleosome is 204 kD and is composed of 108 kD protein and 146 bp DNA. The average mass of all the nucleoprotein particles in the bulk fraction is 203 ± 5 kD, the mode being 204 kD (Table II). This value is within the error of measurement of the mass of the canonical nucleosome and it also agrees with mass measurements of mononucleosomes and oligonucleosomes from chicken erythrocyte nuclei obtained with the STEM(20,21). The mass

Table II. Total mass, DNA and protein content of nucleosomes from chicken erythrocyte

A. Characteristics of nucleosomes in bulk chromatin:			
	<i>Total Mass</i>	<i>DNA Content</i>	<i>Protein Mass</i>
Canonical Nucleosome:	204 kD	146 bp	108 kD
This Study:	203 ± 5	137 ± 3	111 ± 4
B. Characteristics of circular profiles in transcriptionally competent and bulk chromatin:			
	<i>Total Mass</i>	<i>DNA Content</i>	<i>Protein Mass</i>
Competent:	205 ± 4 kD	146 ± 3 bp	112 ± 6 kD
Bulk:	225 ± 8	152 ± 7	123 ± 9
C. Characteristics of u-particles and circular profiles in the transcriptionally competent fraction:			
	<i>Total Mass</i>	<i>DNA Content</i>	<i>Protein Mass</i>
Circular Profiles:	$*205 \pm 4$ kD	146 ± 3 bp	$*104 \pm 4$ kD
U-particles:	$*170 \pm 9$	135 ± 8	$*82 \pm 6$
D. Characteristics of mononucleosome fractionated from chicken erythrocyte nuclei.			
	<i>Total Mass</i>	<i>DNA Content</i>	<i>Protein Mass</i>
	225 ± 4 kD	134 ± 8 bp	116 ± 5 kD

The * indicates that the difference is statistically significant.

histogram is characterized by a broad distribution, perhaps arising from subpopulations (Figure 6), agreeing with previous reports(20,21). The particles represented in the tails of the distribution may not be artifacts of the preparative or measuring procedures but may represent real complexes containing more or less histone or DNA, or nucleosomes in association with other non-histone chromosomal proteins.

Other features of chromatin structure and composition can be described when the protein and DNA components of the total mass of the particles are analyzed separately. ESI uniquely provides the ability to determine these two parameters of individual complexes spread on an EM grid by virtue of the ability to detect phosphorus. The average DNA content of nucleoprotein particles from bulk chromatin is 137 ± 3 bp (Table II), representing a 6% underestimation relative to the canonical nucleosome but well within the estimated margin of experimental error of 10%. Protein content was determined by subtracting the mass arising from the DNA from the total mass. The mean

protein content from the bulk fraction was 111 ± 4 kD (Table II), again agreeing, within the experimental error, with the canonical value of 108 kD. Histograms of both DNA and protein content resembled the histogram of total mass in that subpopulations may be represented (Figure 6). Indeed, a frequency profile of DNA content against total mass (Figure 7) of total chromatin nucleosomes illustrates the variety of nucleoprotein structures present. As expected the majority of structures show DNA content and total mass similar to the canonical nucleosome. But some particles possess low DNA content yet high total mass and vice-versa. This result, incidentally, indicates that mass density effects are not severe and do not limit this analysis (7, 14). In order to better characterize the structures present in chicken erythrocyte chromatin we examined the salt-soluble chromatin fraction and compared it to the bulk chromatin. Mass, phosphorus content and protein content histograms of salt-soluble chromatin subunits are also shown in Figure 6.

Figure 3. Phosphorus enhanced images (160 eV energy loss) of oligonucleosomes from the salt-soluble chromatin fraction. Examples of subunits of circular profiles with reduced mass in the centres are indicated by long arrows and u-shaped subunits are indicated with short arrows. An elongated form is indicated with an arrowhead in (f). Scale bar represents 13 nm.

Figure 4. Phosphorus enhanced images (160 eV energy loss) of selected subunits from oligonucleosome strands from the salt-soluble chromatin fraction. Rows (a) and (b) represent the subunits of circular profile, row (c) of u-shape and row (d) of elongated form. Scale bar represents 7 nm.

Figure 5. (a) Phosphorus enhanced image of an oligonucleosome strand from the salt-soluble chromatin fraction. (b) A region of (a) at $2\times$ the magnification. The arrows indicate where the linkers are thought to terminate at the core particles. Scale bar represents 11 nm.

Comparison of Nucleosomes in Bulk and Salt-Soluble Chromatin

In Figure 8 we show an image of salt-soluble fraction chromatin exhibiting structures typical of the ones from which we derived our values for mass, DNA and protein content, presented in the accompanying table (Table III). The most prevalent morphology in both fractions is described by a circular profile of outer

diameter 10–13 nm and with a reduced mass density in the centre, these representing 89% of the particles in the bulk and 66% in the salt-soluble competent fraction. Circular profiles in the salt-soluble chromatin fraction have a lower average total mass than their counterparts in the bulk fraction (Table II), 205 ± 4 kD compared to 225 ± 8 kD. This difference in mass, however, is not significant at the $p=.05$ level.

Non-circular profiles of nucleoprotein particles were also

Figure 6. Histograms of total mass, phosphorus content and protein mass of subunits from bulk chromatin, from the salt-soluble fraction, and from chicken erythrocyte mononucleosome core particles.

observed, most of these exhibiting a u-shaped appearance (Figures 3 and 4). This morphology has been reported previously in unfixed, H1-containing chromatin prepared from calf thymus (16) though the frequency at which these structures appeared was not stated. We have also observed similarly-shaped structures with reduced protein mass in yeast chromatin spread by the Miller technique (14). Though u-shaped particles have been observed in these other studies, it is impossible to be certain whether the u-shaped complexes prepared from these three different sources are biochemically identical. In this study, the u-shaped particles appeared almost exclusively in the salt-soluble chromatin fraction, accounting for about 22% (16/73) of the subunits in this fraction but only 2% (3/90) of the subunits in the bulk fraction. Thus the u-shaped structures may represent a functionally distinct component of nucleoprotein structures in the salt-soluble transcriptionally competent gene-enriched chromatin fraction. A basis for a functional distinction may lie in the fact that u-shaped structures contain the same amount of DNA, 135 ± 8 bp compared to 146 ± 3 bp, but have significantly less protein mass than do the particles characterized by a circular profile, 82 ± 6 kD compared to 104 ± 4 kD (Table II). A second morphologically

distinct component enriched 3-fold in the salt-soluble chromatin relative to bulk chromatin was an elongated nucleoprotein structure, up to 19.7 nm in length and 5.9 to 8.5 nm in the shorter dimension. These are composed on average of 117 ± 5 bp DNA and 85 ± 4 kD protein. Because of the similarity in mass and DNA content these may represent elongated or straightened u-particles (Figure 4d) and not side views of the more massive particles that frequently display a circular profile. Frequently, the u-shaped structures were not separated by particles of circular profile but occurred in clusters of two or three (Figure 3b), 56% (9/16) of the u-shaped particles occurred in such clusters. The remaining class of particles in the salt-soluble chromatin fraction is characterized by a circular profile of outer diameter equivalent to the nucleosome's but of a lower mass density. An example of such a complex is labelled 6 in Figure 8. From the mass image (left), a visual inspection indicates that it has a lower mass than the neighbouring particles labelled 5 or 7. The difference between object 6 and its neighbours is less pronounced in the phosphorus enhanced image (right). Measurements confirm the visual impression, indicating that the object has the DNA content of a canonical nucleosome but has 20% less protein (see Table III).

We suspect that this class represents an alteration of complete nucleosomes on the basis of their diameters and DNA content, but have no direct evidence to classify them as such. The alternative is that they represent a complex of DNA with a nonhistone chromosomal protein(s), a protein capable of compacting DNA in a manner similar to the nucleosome.

We believe that the classes of structures described represent physiologically relevant states of nucleoprotein particles. It is unlikely that these structural features arise as preparative artifacts either at the stage of cell and nuclei manipulation or at the stage of spreading onto EM grids for the following reasons. First, a

an energy loss of 160 eV, where the phosphorus enhanced image was recorded. The calculation of the reference image was based on the inverse power relationship of spectral intensity and energy loss (see Materials and Methods). The net phosphorus content of eight nucleosomes from one oligomer in the salt-soluble chromatin fraction is presented in Table IV. A comparison of the values using a single reference image (one parameter) and two reference images (two parameter), indicates that similar values are obtained using both methods. We conclude that multiple scattering does not seriously affect the one parameter calculation for specimens in this range of thickness.

DISCUSSION

Susceptibility of DNA to nuclease attack or to base modifications by small molecules has provided insight into the nature of interactions of protein complexes with DNA. Often information at base pair resolution in one dimension can be achieved. The limitation in not providing 3-D information can be overcome entirely if the complex can be crystallized and examined at near atomic resolution by x-rays. When this is not possible other physical techniques such as electron microscopy have been employed instead. More recent advances in electron microscopy, however, have provided the molecular biologist with the ability

to determine simultaneously both structural and chemical information. With electron spectroscopic imaging we have detected morphological variation in the salt-soluble polynucleosomes isolated from chicken erythrocyte nuclei, a fraction enriched in transcriptionally competent DNA sequences. Furthermore, the ability to quantitate mass and phosphorus levels by ESI in individual chromatin subunits of this fraction has provided stoichiometric information of both DNA and protein content.

The salt-soluble polynucleosome fraction from chicken erythrocyte nuclei has previously been described and has been shown to be enriched in β -globin (approximately 50-fold), histone H2A.F, and histone H5 DNA sequences. Furthermore, other characteristics of competent domains such as elevated levels of histone acetylation, ubiquitination of histones H2A and H2B and depletion of histone H1 are descriptive of this fraction. However, the majority of subunits are morphologically identical in both fractions. These structures have circularly shaped profiles and often have reduced mass in the centres, particularly evident in the phosphorus enhanced images. The average values of protein and DNA content of these doughnut-shaped particles in transcriptionally competent chromatin are indistinguishable from their counterparts in bulk chromatin. Additionally, these circular profiles are similar in mass and morphology to the exogenously applied chicken erythrocyte core particles used as mass and phosphorus standards. This preferred orientation on the supporting substrate is evidence for the nucleosome being disc-shaped and having flat faces (22), in contrast to the prolate ellipsoid model proposed by Burlingame *et al* (23).

Nearly 90% of the chromatin subunits in the bulk fraction have this circular morphology, whereas, in the salt-soluble chromatin fraction the particles displaying a circular profile constitute only 66% of the repeating subunits. 30% of the particles are best described as U-shaped or elongated. Besides displaying an altered morphology, these particles are lower in molecular weight, only 170 ± 9 kD instead of 205 ± 4 kD. Because the amount of DNA in the two distinct populations is not significantly different, the lower molecular weight is due to a 20% reduction in the protein content. It has been reported that nucleosome particles that are capable of interacting with RNA polymerase II are deficient in an H2A-H2B dimer(3). Additionally, MNase digestion studies are consistent with the proposal that the β -globin gene chromatin is depleted in H2A-H2B dimers (24). More recently, it has been shown that rearrangements of histones in the core particle occur in nucleosomes that cover the transcribed portion of the hsp 70 gene in heat shocked cells (25). Of the core histones, it is H2A and H2B that are most affected. Though we have not shown it directly, it may be that the lower protein mass is due to the loss of H2A or H2B or the dimer. Alternatively, modification of the histones and/or depletion of the linker histones H1/H5 may account for the 20% reduction in the protein content. Modifications of the histones may increase the mobility of the N-terminal tails of histones H2A, H2B, H3, and H4 and the C-terminal tail of H2A(13,26). The u-shaped nucleosomes may contain hypermodified histones which in turn may alter their structure such that the mobile tails are not accounted for in the protein mass. The depletion in the protein mass of the u-shaped nucleosomes in the salt-soluble chromatin fraction is also quantitatively consistent with the loss of H1/H5 being responsible for the observed differences between the salt-soluble and the bulk chromatin. It is conceivable that the histone acetylation alters nucleosome structure, preventing binding of H1 or H5 (27–30). Moreover, the mass measurements presented here can only detect

Table III. Total mass and DNA and protein content of subunits from an oligomer in the transcriptionally competent fraction

Object	Total Mass (kD)	Basepairs DNA (bp)	Protein Mass (kD)	Type
1	209	155	105	1
2	214	155	111	1
3	169	119	89	2
4	213	143	118	1
5	214	178	96	1
6	186	160	80	4
7	192	144	96	1
8	183	134	93	2
9	128	107	57	3
10	165	130	79	2
11	181	133	93	2
12	194	147	96	1
13	176	141	81	2
14	175	120	95	1
15	241	145	144	1
16	197	149	97	1
17	195	120	114	1
18	261	175	144	1

Type 1 = circular profile

Type 2 = u-particle

Type 3 = low total mass subunit

Type 4 = low protein mass subunit

Table IV. Phosphorous content (b.p.) of oligomeric nucleosomes from salt-soluble chromatin fraction

One parameter calculation	Two parameter calculation
143	123
138	128
169	200
133	118
123	133
133	133
128	107
97	92

a net change in protein mass and cannot distinguish between an unaltered nucleosome and a nucleosome that has lost histone and gained an equivalent mass by interacting specifically or nonspecifically with a nonhistone chromosomal protein. Recently, the observation was made that unfixed hyperacetylated nucleosomes could be disrupted when spread onto EM carbon support films under low ionic strength conditions, a phenomenon not observed if spread on carbon films at higher salt concentrations (31). The apparent disruption gave rise to elongated structures, rather than objects of circular profile. In contrast to the U-shaped particles discussed in this paper, the net mass of these elongated nucleosomes was not reduced. Hence, we do not think that the U-shaped particles are simply nucleosomes containing hyperacetylated histones.

Nevertheless, the loss of protein from the subunit correlates with a change in morphology from a circular to either a U-shape or elongated profile. We propose that if modifications or histone loss alter the stability of the core particle, it may no longer remain a flat disc structure in solution and when fixed and spread onto a EM support substrate the preferred orientation shows these morphologically distinct particles. The u-shaped appearance is possibly a side view of a more open nucleosome complex. This less stable and possibly more open structure would possibly allow the passage of the polymerase enzyme with a minimum amount of histone loss or displacement.

We suggest that the u-shaped particle and an elongated form of equivalent DNA and protein content represent nucleoprotein complexes that are functionally unique with respect to transcription. The content of these unique structures in the salt soluble over the bulk fraction (30% vs. 2%) correlates with the levels of modified histones in these two fractions (Table I). Comparing the acetylation state of the histones in the salt-soluble to that of bulk chromatin, 21% vs. 4% of histone H4 is highly acetylated (tri- and tetra-acetylated forms), and 24% vs. 2% of histone H2B is di-, tri-, or tetra-acetylated. The experiments presented here do not show that the rearranged structures are the direct result of these modifications, though the correlation of the levels of these structures with biochemical modifications is consistent with such an interpretation. The use of specifically labelled antibodies directed against acetylated histones, for example (32,33), represents one approach to answer this question.

The u-shape or elongated structures frequently occur in arrays of two or three, thus occupying up to several hundred base pairs. These structurally modified nucleosomes may function by preventing the stable condensation of the 30 nm chromatin fibril. Solubility of polynucleosomes may result from a sufficient number of contiguous nucleosomes (that is, a run or a string of nucleosomes) which are altered from the canonical form, to prevent the formation of salt precipitable structures. The mathematical model for chromatin fibre folding (34), for example, assumes that a string of a number of contiguous nucleosomes having normal association with linker histones is necessary for the transition to higher order structure. Predictions from the model, when fitted to data, suggested that a run of five to seven contiguous nucleosomes are needed for the transition to higher-order structure. The salt solubility property of competent chromatin may lie in periodic perturbations of 'foldable' chromatin structure. As long as too few contiguous nucleosomes are not of the canonical structure, which allows for normal transitions to higher order structure, precipitation is unlikely to occur. Alternatively it is possible that u-particles represent a subset of nucleosomes capable of exposing a specific region of DNA to transcriptionally important regulatory factors.

Neighbouring nucleosomes with an appearance more characteristic of those in the bulk fraction and of protein mass equivalent to the canonical nucleosome may undergo structural changes of a more transient nature, perhaps only when challenged by a processive enzyme.

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REFERENCES

1. Finch, J.T., Lutter, L.C., Rhodes, D., Brown, R.S., Rushton, B., Lewitt, M., Klug, A. (1977) *Nature* **269**, 29–36.
2. McGhee, J.D., Felsenfeld, G. (1980) *Ann. Rev. Biochem.* **49**, 1115–1156.
3. Baer, B.W., Rhodes, D. (1983) *Nature* **301**, 482–488.
4. Weintraub, H., Worcel, A., Alberts, B. (1976) *Cell* **9**, 409–417.
5. Van Holde, K.E. (1989) *Chromatin*. Springer-Verlag N.Y..
6. Bazett-Jones, D.P., Brown, M.L. (1989) *Mol. Cell. Biol.* **9**, 336–341.
7. Bazett-Jones, D.P. (1988) *J. Ultrastruct. Molec. Struct. Res.* **99**, 59–69.
8. Harauz, G., Ottensmeyer, F.P. (1984) *Science* **226**, 936–940.
9. Bazett-Jones, D.P., Ottensmeyer, F.P. (1982) *Canad. J. Biochem.* **60**, 364–370.
10. Korn, A.P., Spitnik-Elson, P., Elson, D., Ottensmeyer, F.P. (1983) *Eur. J. cell Biol.* **31**, 334–340.
11. Ridsdale, J.A., Davie, J.R. (1987) *Nucl. Acids Res.* **15**, 1081–1096.
12. Ridsdale, J.A., Rattner, J.B., Davie, J.R. (1988) *Nucl. Acids Res.* **16**, 5915–5926.
13. Oliva, R., Bazett-Jones, D.P., Mezquita, C., Dixon, G.H. (1987) *J. Biol. Chem.* **262**, 17016–17025.
14. Bazett-Jones, D.P., Locklear, L., Rattner, J.B. (1988) *J. Ultrastruct. Molec. Struct. Res.* **99**, 48–58.
15. Oudet, P., Gross-Bellard, M., Chambon, P. (1975) *Cell* **4**, 281–300.
16. Langmore, J.P., Wooley, J.C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2691–2695.
17. Kefalas, P., Gray, F.C., Allan, J. (1988) *Nucl. Acids Res.* **16**, 501–517.
18. Drew, H.R., Calladine, C.R. (1987) *J. Mol. Biol.* **195**, 143–173.
19. Woodcock, C.L.F., Frado, L.L.Y., Wall, J.S. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4818–4822.
20. Langmore, J.P., Wooley, J.C. (1977) *Biophys. J.* **17**, 115a.
21. Sewell, B.T., Bouloukos, C., von Holt, C. (1984) *J. Microscopy* **136**, 103–112.
22. Richmond, T.J., Finch, J.T., Rushton, B., Rhodes, D., Klug, A. (1984) *Nature* **311**, 532–538.
23. Burlingame, R.W., Love, W.E., Wang, B.C., Hamlin, R., Xuong, N.H., Moudrianakis, E.N. (1985) *Science* **228**, 546–553.
24. Sun, Y.L., Yuan, Z.X., Bellard, M., Chambon, P. (1986) *EMBO J.* **5**, 293–300.
25. Nacheva, G.A., Guschin, D.Y., Preobrazhenskaya, Karpov, V.L., Ebralidse, K.K., Mirzabekov, A.D. (1989) *Cell* **58**, 27–36.
26. Smith, R.M., Rill, R.L. (1989) *J. Biol. Chem.* **264**, 10574–10581.
27. Norton, V.G., Imai, B.S., Yau, P., Bradbury, E.M. (1989) *Cell* **57**, 449–457.
28. Walker, J., Chen, T.A., Sterner, R., Berger, M., Winston, F., Allfrey, V.G. (1990) *J. Biol. Chem.* **265**, 5736–5746.
29. Perry, C.A., Annunziato, A.T. (1989) *Nucl. Acids Res.* **17**, 4275–4291.
30. Ridsdale, J.A., Hendzel, M.J., Delcuve, G.P., Davie, J.R. (1990) *J. Biol. Chem.* **265**, 5150–5156.
31. Oliva, R., Bazett-Jones, D.P., Locklear, L., Dixon, G.H. (1990) *Nucl. Acids Res.* **18**, 2739–2747.
32. Pfeffer, U., Ferrari, N., Vidali, G. (1986) *J. Biol. Chem.* **261**, 2496–2498.
33. Lin, R., Leone, J.W., Cook, R.G., Allis, C.D. (1989) *J. Cell Biol.* **108**, 1577–1588.
34. Graziano, V., Gerchman, S.E., Ramakrishnan, V. (1988) *J. Mol. Biol.* **203**, 997–1007.