Volume 3 no.9 September 1976 Nucleic Acids Research

Preparation and physical characterization of a homogeneous population of monomeric nucleosomes from HeLa cells

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Received 7 May 1976

ABSTRACT

We describe a method of isolating a homogeneous population of "trimmed" monomeric nucleosomes from Hela cells. These nucleoprotein particles contain a 140 ± 5 base pair length of DNA and have a histone/DNA ratio of 1.2. They lack H1 and contain equal amounts of the four smaller histones. The DNA contains no single strand nicks. The particles sediment with an $S_{20,w}$ of 11S in D_2O density gradients. After formaldehyde fixation, they band at a density of 1.4370 in neutral CsCl. Digestion of nucleosomes with either micrococcal nuclease or DNase I generates the same pattern of DNA fragments observed when intact nuclei are digested. Circular dichroism spectra indicate that the 280 nm positive ellipticity maximum of nucleosomes is about one-half that of chromatin. In the presence of 6 M urea, nucleosomes sediment with an $S_{20,w}$ of 6S, have a multiphasic thermal denaturation profile, and exhibit a circular dichroic spectrum nearly identical to that of B-form DNA. Our yield of purified nucleosomes (10-15% of the input DNA) is similar to the yields of other methods; our nucleosome population is substantially more homogeneous than those previously reported.

INTRODUCTION

Current evidence strongly supports the concept that most, if not all, of chromatin has a subunit structure (for review, see reference 1). Most models for the chromatin subunit contain nucleoprotein particles, termed ν bodies[2] or nucleosomes[3], each consisting of a protein (histone) core around which is wrapped a portion of the DNA fiber. Knowledge of the structure of the nucleosome is of fundamental importance not only for understanding the protein-nucleic acid interactions leading to the packing of DNA within the nucleus, but also for understanding the functional properties of chromatin, leading to the selective expression of genetic information. Most methods for preparing nucleosomes employ nuclease digestion of either nuclei or chromatin. Variations in the reported length of nucleosome DNA may reflect differences in both the amount of nuclease digestion of bridge regions between nucleosomes and the amount of histone H1 associated with the nucleoprotein[4,5]. Such differences, as well as the presence of single-strand nicks

within the nucleosome DNA, would presumably affect the physicochemical properties of the nucleosome population. We therefore have devised a reproducible method for isolating from Hela cells a homogeneous population of trimmed nucleosomes (nucleosome cores). We present the method and compare several physicochemical properties of these nucleosomes with those of both chromatin and nucleosomes prepared by other workers.

EXPERIMENTAL SECTION

Hela S3 cells were maintained in exponential growth in suspension in Eagle's minimum essential medium containing 6% horse serum, penicillin (50 units/ml), and streptomycin (50 µg/ml). For labeling, cells were grown for 22 hrs in medium containing 5-fluorouracil (1×10^{-5} M), thymidine (8×10^{-6} M), and ^{3}H(methyl)thymidine (1mCi/l). Cells were harvested by centrifugation, washed with calcium and magnesium-free Hank's balanced salt solution and either used immediately or frozen until use. We detected no differences between fresh and frozen cells. Nuclei were prepared by suspending cells in 5-10 volumes of ice-cold buffer A[0.25 M sucrose-3 mM $CaCl_2$-10 mM Tris-HCl, pH 8] containing 1% Triton X-100[6] and homogenizing with 15 strokes of a loose-fitting glass Dounce homogenizer. The homogenate was centrifuged at 3000 g for 2 min and the nuclear pellet was washed once with buffer A without Triton X-100.

Chromatin was prepared by a modification of the method of Noll et al[7]. Nuclei were washed once with digestion buffer [0.25 M sucrose-0.1 mM $CaCl_2$-1 mM Tris-HCl, pH 8] and suspended in this buffer at 40 A_{260} units/ml. The suspension was warmed in a water bath at 37° for 4 min and then digested at 37° with micrococcal nuclease (50 units/ml) (Worthington Biochemical Corp) for 2 min. The digestion was stopped by adding 1/300th volume of 0.1 M Na_2 EDTA and chilling. The digested nuclei were recovered by centrifugation at 3000 g for 2 min. The supernatant, containing 15-20% of the input A_{260}, was discarded. The pellet was allowed to swell in ice-cold 0.2 mM Na_2EDTA, pH 8, for 30-60 min with occasional homogenization in a tight-fitting glass Dounce homogenizer. The RNA/DNA ratio of this chromatin is about 0.25; the protein/DNA ratio is about 3.2.

The chromatin concentration was adjusted to 50 A_{260} units/ml with 0.2 mM Na_2EDTA. 5 M NaCl was added with stirring to a final concentration of 0.15 M, 1/300th volume of 0.1 M $CaCl_2$ was added, the suspension was warmed at 37° for 4 min and digested at 37° with micrococcal nuclease (100 units/ml) for 8-16 min. The digest was made 1 mM in Na_2EDTA, chilled, and centrifuged at 6000 g for 5 min. The supernatant, containing 20-30% of the input A_{260},

was layered over isokinetic sucrose gradients containing 1 mM Na_2EDTA and 0.1 M NaCl, with a meniscus concentration of 5% (w/w) sucrose. Centrifugation was for 16 hr. at 4° at 27,000 rpm in the SW 27 rotor (Beckman). Gradients were emptied by pumping from the bottom of the tube through a DB-G spectrophotometer equipped with a flow cell and log recorder. The nucleosome peak was collected and analyzed as described.

Thermal denaturation measurements and circular dichroism spectra were obtained for nucleosomes which had been dialyzed extensively against 0.25 mM Na_2EDTA, pH 7, as previously described[8,9]. Analytical isopycnic banding was in 45% CsCl at 44,000 rpm and 25° in the Model E Ultracentrifuge (Beckman) equipped with an automatic photoelectric scanner system. Velocity sedimentation measurements were made using the self-generating density gradient system described by Vinograd et al[10] with D_2O as the solvent.

For nuclease digestion, nucleosomes were suspended at about 1.5 A_{260} units/ml in the following buffers: micrococcal nuclease - [0.1 mM $CaCl_2$-5 mM Tris-HCl, pH 8]; DNase I -[10 mM $MgCl_2$ - 5 mM Tris-HCl, pH 8]; S_1 nuclease-[1 mM $ZnSO_4$ - 50 mM Na acetate, pH 5].

DNA was purified from nucleosomes by phenol extraction, analyzed in polyacrylamide gels, using the buffer system of Peacock and Dingman[11], and stained with "Stains-All", as previously described[12]. The length of the DNA fragments was determined by the method of Maniatis et al[13]. Gels were scanned with an E-C Densitometer.

For protein analyses, nucleosomes were made 1-2% in SDS, dialyzed overnight into sample buffer, and electrophoresed in discontinuous SDS-polyacrylamide gels using the buffer system of LeStourgeon and Rusch[14]. The separating gel contained 18% acrylamide - 0.16% bisacrylamide; the stacking gel contained 3% acrylamide - 0.08% bisacrylamide. Gels were stained for one hour in 0.2% Coomassie Blue in 50% methanol - 7% acetic acid and destained in 20% methanol - 7% acetic acid. Histones were extracted by making nucleosomes 0.4 N in ice-cold H_2SO_4 and stirring for 60 min. The suspension was centrifuged at 6000 g for 15 min; the supernatant was removed and dialyzed extensively against water. Protein concentrations were determined by the method of Lowry et al[15] using calf thymus histones as standards. RNA was measured by the method of Fleck and Munro[16].

RESULTS

Our procedure for preparing a homogeneous nucleosome population involves four basic steps. First, we prepare chromatin by briefly digesting Hela nuclei with micrococcal nuclease in low salt and then allowing the

nuclei to swell in hypotonic EDTA. This procedure minimizes the mechanical shearing to which the DNA is exposed, and the DNA retains its native susceptibility to the nuclease. Second, the chromatin is then further digested with micrococcal nuclease in the presence of 0.15 M NaCl. At physiologic salt concentrations chromatin has a susceptibility to nuclease digestion similar to that at low salt concentrations; furthermore, during digestion in the presence of 0.15 M NaCl, the generation of nucleosomes is increased somewhat, apparently due to a decrease in their further digestion to smaller fragments (data not shown). Third, the digested chromatin is centrifuged briefly, and the supernatant is layered on preparative sucrose gradients. Although both the supernatant and the pellet contain approximately equal quantities of nucleosomes, the supernatant contains virtually no higher-order subunits (dimers, trimers, etc.), whereas the pellet contains a substantial amount of these structures. Thus, the brief centrifugation produces an initial purification of nucleosomes. Furthermore, the supernatant contains no detectable histone H1; essentially all of the H1 appears in the pellet. The absence of H1 greatly lessens the tendency of nucleosomes to aggregate during centrifugation. Fourth, the preparative sucrose gradients contain 0.1 M NaCl; this salt concentration increases the resolving power of the gradients, as compared to gradients containing either no NaCl or 0.5 M NaCl (Fig. 1). The reason(s) for this are unknown; pre-

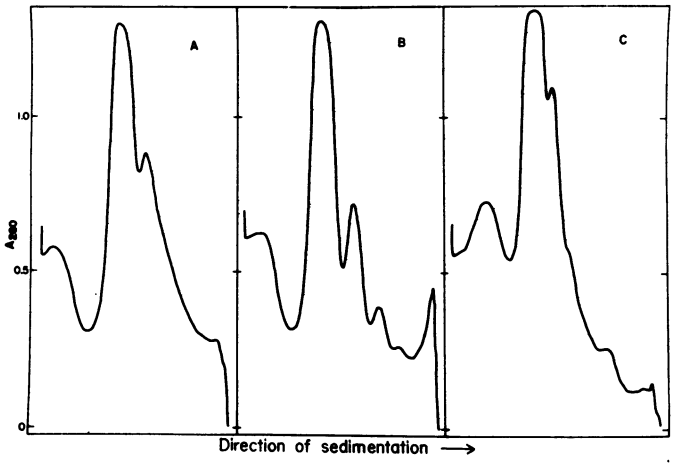

Figure 1. Effect of salt on the sedimentation of nucleosomes. Chromatin was digested for 3 min with 100 units/mL of micrococcal nuclease and centrifuged briefly; the supernatant was layered over isokinetic sucrose gradients as described in the Experimental Section. The gradients also contained (A) no added NaCl; (B) 0.1 M NaCl; (C) 0.5 M NaCl.

sumably, nucleoprotein particles tend to aggregate in low salt, primarily through electrostatic interactions; in the presence of 0.1 M NaCl, these non-specific interactions are decreased. As the salt concentration in the gradient is raised to 0.5 M NaCl, the further reduction in electrostatic interactions is presumably outweighed by the simultaneous stabilization of hydrophobic interactions, again leading to non-specific aggregation of the nucleoprotein particles. Woodcock et al[17] observed maximal resolution only when the salt concentration in their sucrose gradients was increased to 0.5 M. Presumably, this is due to the presence of histone H5 in their preparations. A typical preparative sucrose gradient contains a nucleosome peak and a more slowly-sedimenting population of small nucleoprotein fragments. The amount of material in the nucleosome peak remains relatively constant over a 4 to 5-fold range of chromatin digestion; the amount of slowly-sedimenting material increases as digestion increases, suggesting that the rate of production of nucleosomes equals the rate of their degradation during digestion. The yield of purified nucleosomes represents 10-15% of the DNA of the starting material (Table 1).

TABLE I

Fraction	TCA-insoluble ^{3}H-thymidine (cpm) x 10^{-6}	% of starting material
Nuclei	45.5	100
1st Supernatant	2.7	6
Chromatin	41.6	91
2nd Supernatant	8.8	19
Nucleosomes	6.2	14

The DNA isolated from nucleosomes migrates in polyacrylamide gels as a single band and has a length of 140 ± 5 base pairs (Figure 2). Under denaturing conditions, the DNA also migrates as a single band, indicating that it contains no single strand nicks (Figure 3).

The nucleosomes contain each of the four smaller histones in approximately equal quantities and no histone H1 (Figure 4). The total protein/DNA ratio of nucleosomes is 1.9 - 2.1 when measured by the method of Lowry et al. This value was confirmed by fixing nucleosomes in formaldehyde and sedimenting them to equilibrium in cesium chloride; the buoyant density, 1.4370, indicates a protein/DNA ratio of 1.95, assuming a partial specific volume of 0.74 for the protein. These results suggest that, in addition to the

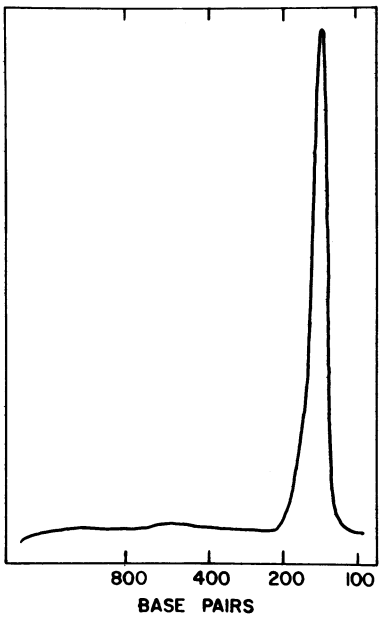

Figure 2. Length of nucleosome DNA. DNA was purified by phenol extraction of nucelosomes, analyzed on a 3.5% polyacrylamide gel, and the gel was scanned. The mobility was compared to the mobilities of xylene cyanol FF and bromphenol blue. Ordinate is linear with optical density.

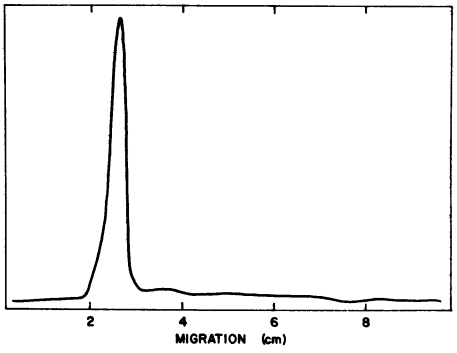

Figure 3. Nucleosome DNA contains no single-strand nicks. Nucleosomes DNA was heated to 100° for 5 mins, quenched in ice, electrophoresed in a 6% polyacrylamide gel containing 6 M urea, and the gel was scanned. Ordinate is linear with optical density.

histones, nucleosomes also contain a lesser amount of non-histone protein. The ratio of acid-extractable protein to DNA is 1.2. This is the expected value for a 140 base pair length of DNA associated with two each of the four smaller histones.

Digestion of nucleosomes with micrococcal nuclease generates a pattern of DNA fragments typical of that produced in a limit digest of nuclei[5,18,19]. This observation indicates that the nucleosomes are a representative population and that we have not selected an atypical nuclease-resistant fraction. Digestion of nucleosomes with DNase I introduces single-strand nicks at ten-base intervals[20]; again this is typical of DNase I activity on intact nuclei[21]. We have been unable to detect any single-strand nicks when nucleosomes are exposed to S_1 nuclease in various concentrations and for various times (data not shown). This suggests that the nucleosome has

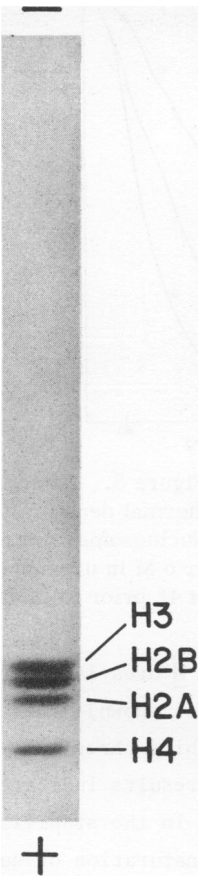

Figure 4. Nucleosome proteins. Proteins from purified nucleosomes were analyzed on discontinous 18% polyacrylamide-SDS gels as described in Experimental Section.

little, if any, single-stranded character. If "kinks"[22] exist in nucleosome DNA, they apparently are not susceptible to digestion by S_1 nuclease.

Nucleosomes sediment as a homogeneous zone during analytical density gradient centrifugation in D_2O at 20°. The apparent sedimentation coefficient at an average DNA concentration of 20 µg/ml was 10.9×10^{-13} sec^{-1}.

Urea produces marked alterations in chromatin structure without dissociating histones from DNA. The nucleoprotein becomes extended, indicated by an increase in its intrinsic viscosity and a decrease in its apparent sedimentation coefficient; the circular dichroism spectrum approaches that of B-form DNA and the melting profile contains a series of defined, separate thermal transitions[23-25]. In 6 M urea, nucleosomes have an apparent sedimentation coefficient of 6.2×10^{-13} sec^{-1}; the circular dichroism

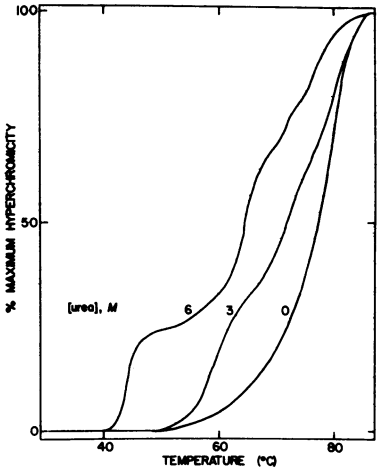

Figure 5. Effect of urea on the thermal denaturation of nucleosomes. Nucleosomes were made 0 M, 3 M, or 6 M in urea and incubated for 20 hr at 4° prior to thermal denaturation.

spectrum from 260-300 mm of nucleosomes in 6 M urea is identical to that of protein-free DNA in the same solvent (data not shown); the thermal denaturation profile of nucleosomes in 6 M urea exhibits three well-defined transitions at 44°, 64°, and 73° (Figure 5). The results indicate that histone-histone interactions play a substantial role in the stabilization of DNA structure in the nucleosome. The thermal denaturation of nucleosomes in the absence of urea is similar to that reported previously[26].

Circular dichroism spectra (Figure 6) indicate that nucleosomes have a conformation different from both chromatin and DNA. Chromatin has a maximum ellipticity about one-half that of DNA; in contrast, nucleosomes have a maximum ellipticity only one-fourth that of free DNA. Chromatin which has been digested with RNase A (10 µg/ml, 37°, 20 min) also has a maximum ellipticity about twice that of nucleosomes (data not shown). These results indicate not only that the DNA within the nucleosome has a substantially altered conformation, compared with free DNA, but imply that whole chromatin contains regions of DNA which are not in the nucleosome conformation. Nuclease-resistant "PS particles" from calf thymus have circular dichroism spectra similar to Hela nucleosomes; however, PS particles differ from nucleosomes with respect to solubility in 0.15 M NaCl, sedimentation coefficient, and thermal denaturation[27,28].

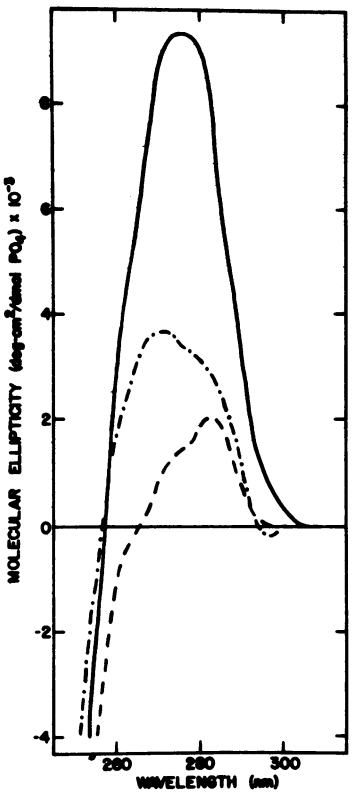

Figure 6. Circular dichroism of chromatin (-.-), native nucleosomes (- - -), and nucleosomes dissociated by the addition of solid SDS to 0.5% (——).

DISCUSSION

Studies of the nucleoprotein structure of several different tissues indicates that the chromatin subunit contains regions having different nuclease susceptibilities[5,12,18,19,29]. Such observations have usually been interpreted in terms of relatively nuclease-sensitive "bridge" regions separating relatively nuclease-resistant "beads". In order to study the organization of the nucleoprotein within these bead regions, we have developed a simple, reproducible method for their preparation as a highly homogeneous intact population.

Our yield of nucleosomes is at least equal to the yields achieved by others; we estimate that most procedures for nucleosome preparation have yields in the range of 10-15%[18,29-32]. The two novel features of our method,

i.e., the digestion of chromatin in 0.15 M NaCl and the inclusion of 0.1 M NaCl in the sucrose gradients, generate a more homogeneous nucleosome population than other reported procedures. For example, where size estimations have been made, the "monomer" fractions described previously contain 185-140 base pair[18], 150-115 base pair[29], and 50-200 base pair[31] lengths of DNA. Thus, our procedure allows us to prepare a substantially more homogeneous nucleosome population, containing a 135-145 base pair DNA fragment, without a substantial decrease in yield. We routinely prepare from a liter of cells about 1 mg of DNA as purified nucleosomes. Scale-up to 30-40 mg should be possible with the use of zonal centrifugation systems.

In the intact cell, the nucleoprotein is exposed to, and functions at, "physiologic" ionic concentrations. We desired, therefore, to isolate nucleosomes under ionic conditions more nearly approximating those in vivo; thus, the inclusion of 0.15 M NaCl in the digestion buffer. We have elected to use chromatin, prepared by a brief nuclease digestion, since we have encountered difficulties in maintaining intact nuclei in a relatively homogeneous suspension at a salt concentration of 0.15 M NaCl. This pre-digestion, during which 5-10% of the DNA becomes acid-soluble, markedly increases the ease with which the remaining chromatin is solubilized. Hancock[33] has indicated that some protein rearrangement may occur when nucleoprotein is exposed to 0.15 M NaCl. On the other hand, essentially no histone protein is extractable from nuclei at 0.15 M NaCl, and the possible rearrangement of non-histone proteins that occur at this ionic strength may reflect physiologic properties of the nucleoprotein.

We think that the purified nucleosomes are representative of native chromatin for the following reasons: 1) they are digestible by micrococcal nuclease, indicating that we have not selected nuclease-resistant material. 2) Extensive digestion of nucleosomes or nuclei by micrococcal nuclease each generates typical limit digest patterns of DNA fragments. Similar observations have been made previously[18,19], and indicate that the nucleosomes retain the susceptibility to digestion of intact nuclei. 3) Both nucleosomes and nuclei are nicked at ten base intervals by DNase I[20], in agreement with the finding of Shaw et al[29], again indicating that the nucleosomes retain the DNA conformation typical of intact nuclei.

In addition to their susceptibility to nucleases, Hela nucleosomes have other properties similar to nucleosomes from a variety of tissues 1) a DNA fragment about 140 base pairs in length[18,29]; 2) no histone H1[4,5,29]; 3) two each of the four smaller histones[34,35]; 4) a lesser amount of non-

histone protein[30,32] and 5) a sedimentation coefficient of about 11S[29,30,31].

The availability of this homogeneous nucleosome population has facilitated a more detailed examination of the internal architecture of the nucleosome; for example, it has allowed us to map the DNase I susceptible sites within the nucleosome[20] and to determine which histones are located near the ends of nucleosome DNA (R. T. Simpson, manuscript in preparation).

ACKNOWLEDGMENTS

We thank Ms. Linda Propst for her photographic assistance and Ms. Joan Shores for typing the manuscript. S_1 nuclease was obtained from Dr. George Rushizky.

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