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PRELIMINARY CHARACTERIZATION OF CHELATION-SENSITIVE NUCLEOPROTEIN PARTICLES

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SUMMARY: Nucleoprotein particles (B2), isolated following digestion of calf thymus chromatin with micrococcal nuclease, are resolved on a non-chelating Bio-Gel A-5m column. B2 protein electrophoresis showed the presence of several H1 species and several nonhistone proteins but was depleted in core histones. DNA electrophoresis demonstrated that native B2 DNA has a length of about 46 base pairs. On DNA sequencing gels, the length distribution of denatured B2 DNA ranged from 12 to 35 bases with a weighted average chain length of about 26 bases. Depletion of a 20 base band in B2 DNA suggested specific protection of internucleosomal DNA sites during the nuclease digestion.

INTRODUCTION: In the eukaryotic genome chromatin is organized into repeating units of nucleosomal core particles composed of protein and DNA interspersed with spacer regions of DNA lacking core proteins (1-4). Other nuclear components contribute to the architecture of the genome *in vivo*; among these constituents are divalent cations. Moreover, the addition of chelating agents following endonuclease digestion of chromatin *in vitro* yields multivalent cation-free nuclear particles differing in composition from "native" chromatin. We are reporting the isolation in the presence of multivalent cations of histone containing nuclear particles which are depleted in core histone proteins and enriched in non-histone proteins; these particles are not readily detected in the solubilized chromatin fraction when chelating agents are used in the procedure.

MATERIALS AND METHODS

Isolation of Nuclei, Nuclease Digestion and Fractionation of Chromatin. Procedures have previously been described (5).

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Isolation of Protein and DNA from Nuclear Fragments. Peak column fractions were pooled and precipitated by the addition of M_gCl_2 and 2 vol of ethanol at -20° (final $MgCl_2$ concentration 1 mM). Isolation of protein was performed by the phenol extraction method described by Le Stourgeon and Beyer (6).

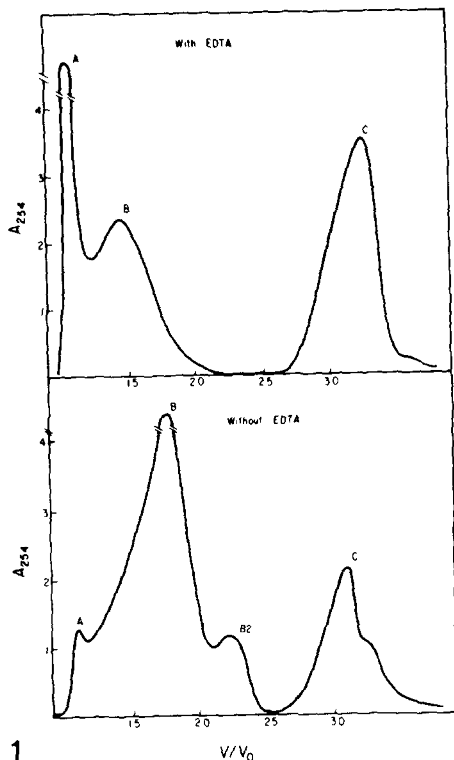
For isolation of DNA the aqueous phases from consecutive phenol extractions were pooled and subjected to digestion with 0.2 mg/ml pronase (Cal. Biochem. nuclease-free) for 15 min in a 37° water bath shaker with mild agitation. Following digestion samples were re-extracted with phenol twice and the DNA precipitated by the addition of M_gCl_2 and ethyl alcohol at -20° as above. DNA from column fraction B2 was isolated by harvesting the alcohol precipitated column fraction (centrifugation), washing with cold ethanol, and resuspending the drained precipitate in 0.1 x SSC on ice. The suspension was then made up to 1 x SSC, 1% SDS, 1 μg/ml pronase, and incubated for several hours at 37°C. The material was centrifuged briefly and the supernatant was precipitated with two volumes of ethanol overnight in the freezer. The DNA was then harvested by centrifugation, ethanol washed, dried and resuspended on ice in DNA electrophoresis buffer.

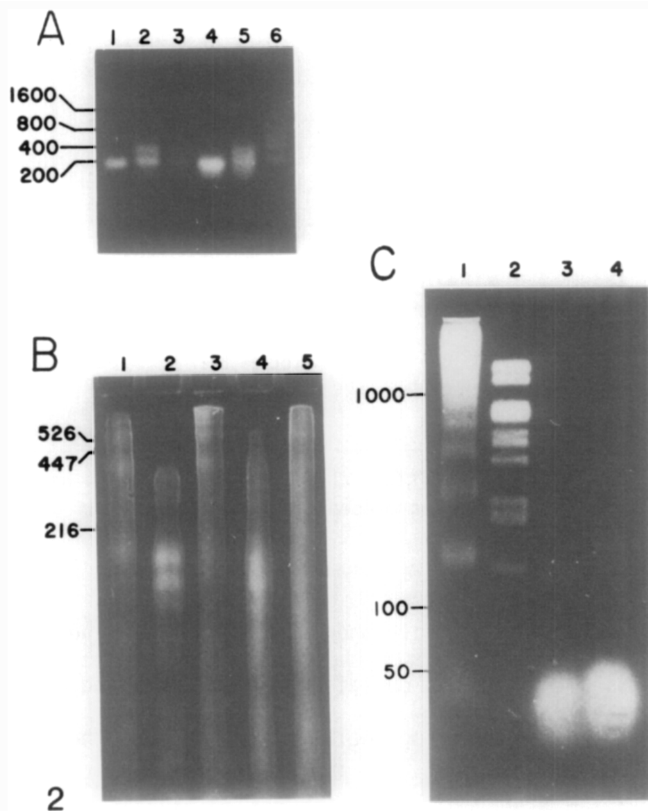
Protein and DNA Electrophoresis and Autoradiography. Protein electrophoresis was performed on vertical 0.75 mm SDS - polyacrylamide (8.75% or 12.5%) slab gels according to the method of Le Stourgeon and Beyer (6). DNA electrophoresis was performed using a number of different systems specified in the figure legends. Samples were loaded onto either flat bed or vertical slab gels in the relevant buffer containing 10% sucrose, or buffered 80% formamide in the case of the DNA sequencing gels. Gels were stained in electrophoresis buffer (50 mM Tris, 50 mM Boric Acid, 1 mM EDTA, pH 8.8) using 5×10^{-7} M 33258 Hoechst (Calbiochem), de-stained and fixed in 5% acetic acid at 4°C. Fluorescent photography was accomplished with black light side illumination, 35 mm plus X film and a yellow #8 filter. For autoradiography, sequencing gels overlaid with plastic wrap and RP-type X-ray film were placed in an X-ray cassette with a Chronex intensifier screen at -70°C.

^{32}P End-Labeling of DNA. Nuclease digested and purified DNA was terminally labeled in the 5' position using a modification of the forward reaction of Lillehaug *et al.* (7) with T4 polynucleotide kinase (Miles) and γ- ^{32}P ATP (Amersham, aqueous triethyl ammonium salt, 3000 Ci/mMole). Removal of the unreacted ^{32}P was accomplished by alcohol precipitation, resuspension in 0.1 M NaOH, neutralizing with Tris buffer, reprecipitating with ethanol, washing with ethanol, drying, and resuspending in buffered formamide.

RESULTS AND DISCUSSION

Chromatin was digested with micrococcal nuclease to 35% solubility, dialyzed against Tris-EDTA, and the supernatant loaded onto a gel filtration column. A typical elution profile (A_{254}) appears in Fig. 1a and agrees with profiles previously published (8). DNA and protein were separated from pooled peak fractions and electrophoretic analysis was performed. Denatured DNA from peak fraction B (the presumptive mononucleosomal peak) was sized at 160 bases, Fig. 2a and Table I, and that from peak A was identified with DNA lengths in the di- to tri-nucleosomal range, but also contained material associated with some higher order oligonucleosomes.





Figures 1a & b: (Left) BIO-GEL A-5m column profiles of calf thymus chromatin digested with Micrococcal nuclease and solubilized with (a) and without (b) EDTA.

Figure 2: (Right) A. Denatured DNA gel composed of 1.5% agarose in 40 mM KOH, stained with Hoechst dye in acidic conditions. Marker DNAs (Hae III PM2 and *Hind III SV-40*) in adjacent slots (not shown) provided the calibration (in bases) to the left of the photograph. The DNA samples are: Slot 1, peak fraction B from the nonchelated column (Fig. 1b); Slot 2, fraction from the nonchelated column (Fig. 1b) having a v/vo 1.5-1.6. Slot 3, peak fraction A from the non-chelated column (Fig. 1b); Slot 4, peak fraction B from the chelated column (Fig. 1a); Slot 5, peak fraction A from the chelated column (Fig. 1a); Slot 6, approximately 10 μg of a nuclease digest of rat chromatin (to a level of 3.3% acid solubility).

B. Denaturing gel composed of 7M Urea, 4% acrylamide (0.4% methylene bis-acrylamide) in 50 mM Tris-borate, 10^{-3}M EDTA, pH 8.3, stained with Hoechst dye in acidic conditions. Hind III SV-40 DNA was used as a marker in an adjacent slot (not shown); the position of the three smallest fragments of the marker DNA (given in bases) are designated to the left of the gel photograph. The DNA samples are: Slot 1, *fraction from the nonchelated column (Fig. 1b)*; Slot 2, peak fraction B from the nonchelated column (Fig. 1b); Slot 3, peak fraction A from the nonchelated column (Fig. 1b); Slot 4, peak fraction B from the chelated column (Fig. 1a). Slot 5, peak fraction A from the chelated column (Fig. 1a).

C. Native gel composed of 0.5% agarose, 2% acrylamide (0.07% methylene bis-acrylamide) 50 mM Tris-borate, 1 mM EDTA, pH 8.3, stained with Hoechst dye under acidic conditions. The DNA samples are: Slot 1, 10 μg of a nuclease digest of rat chromatin (to a level of 3.3% acidic solubility). Slot 2, 4 μg of the *III PM2 DNA* (used as the molecular weight marker). Slot 3, native DNA from peak fraction B2 of the nonchelated column (Fig. 1b). Slot 4, heat denatured DNA from peak fraction B2 of the nonchelated column (Fig. 1b).

Table I: CHARACTERIZATION OF POOLED PEAK FRACTIONS FROM FIG. 1.

Peak	V/Vo	280/260	Protein Core	Protein NHC*	bp DNA Length
WITH EDTA					
A	1.0	.54 ± .05	+	?	≥160
B	1.48 ± .04	.52 ± .06	+	ND	160
C**	3.00 ± .17	.51 ± .04	ND	ND	alch.sol.
WITHOUT EDTA					
A	1.0	.81 ± .08	?	?	≥160
B	1.68 ± .02	.56 ± .06	+	+	160
B2	2.07 ± .02	.54 ± .03	ND	+	<46
C**	2.59 ± .07	.43 ± .04	ND	ND	alch.sol.

*Non-histone chromatin proteins such as HMG
**DNA was present; RNA and protein, if present, was less than 10 μg/ml

Protein electrophoresis using 12.5% and 8.75% polyacrilamide gels indicated that peak B contains core histones and nonhistone proteins (Table I).

Omitting chelating agents from the solubilization procedure and from the column buffer yielded the elution profile shown in Fig. 1b, in which a new peak (B2) appeared. Also, the mononucleosomal peak (B) chromatographed as if it were more compact, which is reasonable in the presence of divalent cations. DNA from peak fraction B again sized at 160 bases (Fig. 2a), and peak fraction A again contained mostly DNA lengths of the di- and tri-nucleosomal-sized fragments. Peak B2 DNA was found to have a mean length of 46 base pairs (Fig. 2c, Table I). Protein electrophoresis of peak fraction B (Figs. 3 and 4, Table I) gave a pattern indistinguishable from the corresponding peak fraction B isolated using EDTA. Electrophoresis of peak B2 protein showed the presence of several H1 species and several nonhistone proteins and reflected a depletion of core histones (Figs. 2 and 4, Table I). Over 20 protein bands have been detected in peak B2. DNP gels (not presented) have also shown B2 peaks to be heterogeneous and distinctive from peak B.

The chromatographic and compositional properties of B2 nucleoprotein suggest an <u>internucleosomal</u> species containing a small segment DNA which has been protected from nuclease attack by H1 and non-histone proteins associa-

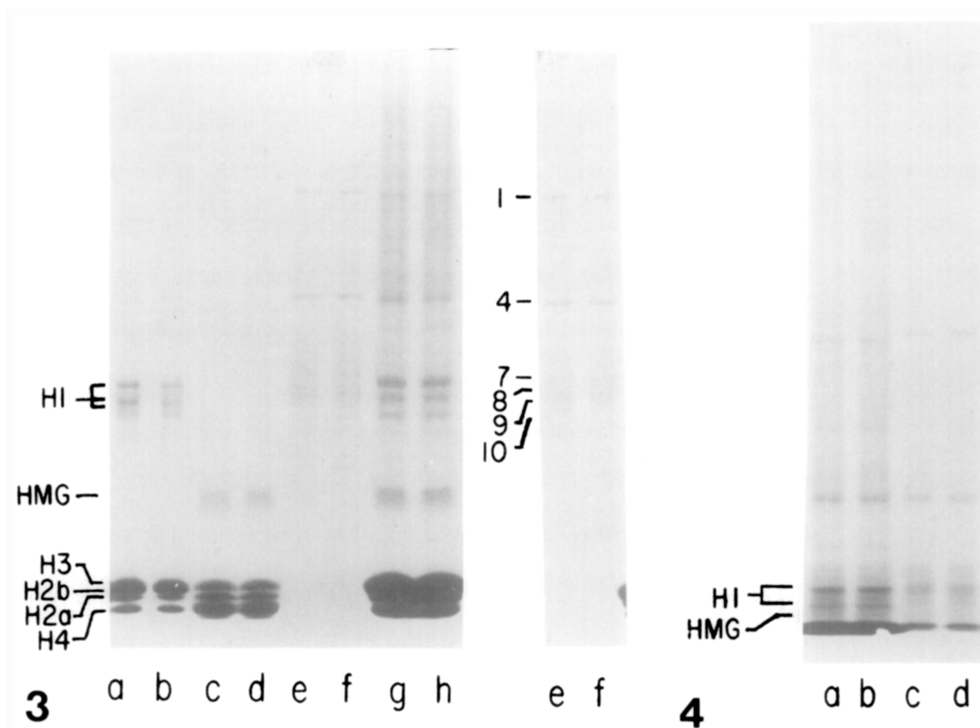


Figure 3. (Left) 12.5% Polyacrylamide gel electrophoresis of proteins extracted from non-chelated BIO-GEL A-5m column peaks (Fig. 1b). (a,b) Total histone standard; (c,d) Column peak B; (e,f) Column peak B2; (g,h) Phenol extract of nuclei. Right: Peak B2 is shown labeling proteins (e,f) determined from electrophoresis of marker proteins. BAND 1; MW 87,500; BAND 8; MW 23,500; BAND 4; MW 42,000; BAND 9; MW 23,000; BAND 7; MW 25,000; BAND 10; MW 22,500.

Figure 4. (Right) 8.75% Polyacrylamide gel electrophoresis of proteins extracted from a non-chelated BIO-GEL A-5m column peaks (Fig. 1b). (a,b) Phenol extract of nuclei; (c,d) Column peak B2.

ted with linker DNA (9-10). Other characterizations of the unusual properties of this chromatin fraction, the most important of which is its sensitivity to chelation are also in support of this identification. For example, gel filtration of undialyzed digest supernatant (immediately centrifuged after 22 minutes of digestion) yielded comparable amounts of peak B2 to confirm that peak B2 was indeed produced during the initial 37°C digestion period. Further studies showed that the size of peak B2 is dependent on the temperature and time of digestion and relative concentration of chelating agents in the presence of nuclease.

Additional information was gained from attempting kinetic analyses of the B2 particle yield. The relative amount of the column fractions as a

function of digestion time was estimated using the non-chelated column profiles similar to that of Fig. 1b. The resolution of the profile components was performed by Gaussian deconvolution using a Dupont electronic curve resolver. The kinetic yield data demonstrated that the temporal production of the B2 column fraction proceeded at a slightly greater rate than that of fraction B for the digestion times over which these experiments were done. This is the expected kinetic result, since monomer (fraction B) is being produced at a diminished rate during this time domain and presumed linker particle (fraction B2) is being rapidly produced in the conversion of the 200 bp to the 140 bp mononucleosome (9). The absorbance of both fractions B and B2 are constantly diminishing during digestion due to loss of their nucleic acid constituents, which is apparent from the temporal diminution of their respective A_{254}/A_{280} ratio. Hence, optical absorbance cannot be unambiguously related to the number of chromatin particles during digestion, and consequently should not be used as the basis for a quantitative kinetic analysis. However, these approximate kinetic analyses do support the picture that B2 is being produced from the linker region and the relative amounts of B and B2 (3 to 5:1, see Fig. 1b) are in approximately the correct ratio.

Adequate chain length resolution of B2 DNA can be obtained using DNA sequencing gels (11). Fraction B and B2 DNAs were end-labeled with polynucleotide kinase and electrophoresed in a DNA sequencing gel (Fig. 5); chain lengths were marked with a DNA that was being sequenced in adjacent slots, and these lengths are noted. Several subnucleosomal species in peak fraction B DNA are clearly delineated, and are, for the most part, larger than those observed by Bakayev *et al.* (14). It seems most likely that these DNA species are products of internal nicking of the nucleosome. As in Fig. 2b, the nucleosomal DNA is clearly resolved into two species, expected from other studies (9-10). A particularly striking feature of the major portion of B2 DNA electrophoretic pattern (Fig. 4) is the depletion of an oligomer size class, that we estimate has either a 20 or 21 base length. The un-

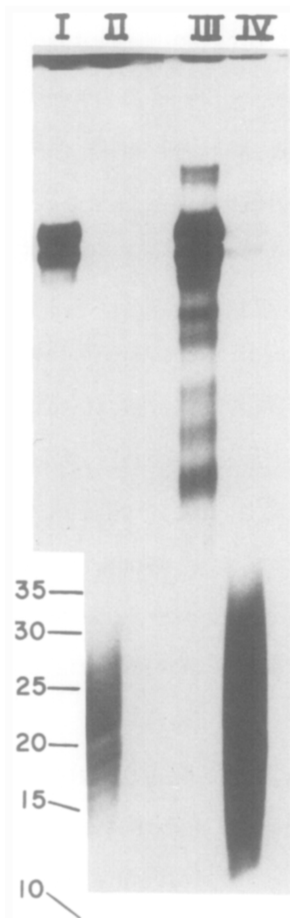


Figure 5: Autoradiographic image of a high resolution (DNA sequencing) gel demonstrating the oligonucleotide length of peak fraction B and B2 DNAs from the non-chelated column (Fig. 1b). The ^{32}P end-labeled DNA was electrophoresed on a 8% acrilamide (0.4% methylene bis-acrilamide) gel in 7 M urea and electrophoresis buffer. Lanes I and II are images of B and B2 DNAs, respectively, and lanes III and IV are overexposed images of the same gel. Lengths in bases are marked and were derived from a DNA that was being sequenced in adjacent slots (not shown).

certainty arises from the excess 3'phosphate on these molecules. The length distribution of the B2 DNA is consistent with the previous estimate from the other gels where the weighted average chain length distribution is about 24 bases, and ranges from 12 to 35 bases; oligomers less than about 12 bases in length were excluded by the isolation technique.

Bakayev *et al.* (12), have explored what they call subnucleosomal particles (meaning smaller than nucleosomal rather than implying part of a nucleosome). Their data suggest that there exists among the several

detected subnucleosomal particles an H1-containing particle possessing a 35 base pair piece of duplex DNA. The B2 nucleoprotein particle discussed in our study, however, has been isolated under different conditions and appears to have somewhat different properties.

Finally, two different observations support a natural rather than manipulative origin of the B2 particles . First the sensitivity of this complex to chelation (5) would contradict the model involving adventitious binding of basic proteins to DNA, since multivalent cations would probably hinder rather than assist such binding. Secondly, and most important, the specific depletion of the 20 base band in the single strand length analysis of B2 suggests a specific protection in a nuclease digestion pattern which must derive from a specific structural attribute of a protein-DNA complex. Also, there is the supportive evidence that the yield of B2 particle happens to be what is expected from the linker region.

We conclude, therefore, that B2 particles reflect upon a real inter nucleosomal chromatin structure, and that such particles are readily isolated chromatographically on a preparative scale. Further characterization of sub-nucleosomal particles is particularly important and may be applicable to the fundamental understanding of eukaryotic chromosomal structure and expresion.

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