Two-Dimensional Electrophoretic Analysis of Polynucleosomes*

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Electrophoretic techniques have been developed to map simultaneously the total distributions of DNA sizes, protein compositions, and nuclease mediated precursor-product relationships present in a heterogeneous population of mono- and polynucleosomes. Chromatin, prepared from nuclei after controlled micrococcal nuclease digestion, is separated by gel electrophoresis at low ionic strength. The DNA or protein components, or both, are displayed next by electrophoresis in a second dimension, using sodium dodecyl sulfate-polyacrylamide slab gels. Mono- and polynucleosomes migrate as molecularly dispersed protein-DNA complexes in the first dimension, while protein and DNA species migrate independently in the second dimension. In order to trace precursor-product relationships, electrophoretically separated mono- and polynucleosomes are digested in situ prior to performing electrophoresis to display DNA in the second dimension. This is accomplished by incorporating reversibly inactivated micrococcal nuclease or DNase I within the first dimension gel matrix and running buffer. The enzymes are reactivated after electrophoresis by exposing gels to the appropriate divalent cations. Such a "chromatin fingerprinting" technique can discriminate between various levels of nucleosome organization with regard to differences in core and spacer DNA lengths. This general strategy could be applied to a variety of other problems, including restriction endonuclease site mapping.

Application of the above procedures to a systematic study of bovine thymus chromatin conclusively demonstrates a spectrum of nuclease processing events, many of which were only supposed to occur previously. Most of the bovine genome is shown to consist of 100 base pair cores which are nuclease products of an average repeat of 191 ± 8 base pairs. These cores are processed further to 140 base pair and smaller DNA fragments. A minor portion of chromatin consists of a larger repeat of 203 ± 9 base pairs which gives rise to two unique classes of mononucleosomes with larger DNA lengths. Other minor classes of repeats exist, with lengths of 170 ± 8 and 142 ± 3 base pairs. In all, five monomer nucleoprotein particles have been identified, and certain aspects of their precursor-product relationships and protein compositions have been established.

Due to pioneering biochemical and electron microscopic studies, it is now clear that a large proportion of the chromatin of eukaryotic cells consists of a flexible chain of subunits, termed ρ bodies, or nucleosomes (3-9). By virtue of the fact that internucleosomal DNA is preferentially hydrolyzed by micrococcal nuclease, nucleosome oligomers and monomers can be released from chromatin after controlled endonuclease treatment. Resulting products have been isolated by sucrose gradient centrifugation or column chromatography and are found to consist of multiples of about 200 base pairs of DNA complexed with chromatin proteins, in particular the histones (6, 10-15). The diameter of nucleosome monomers is close to 100 Å (7-9, 15). DNA is believed to be wrapped around the outside of a histone cluster (17-19), yielding a packing ratio of about 7:1 (20) and a constraint equivalent to an average of greater than one negative superhelical turn (21, 22). At present, there is no evidence that nucleosomes are arranged specifically with regard to DNA base sequence (12, 23-26). It is likely that each nucleosome consists of 8 histone molecules, 2 each of histones H2A, H2B, H3, and H4 (17, 27, 28). It has been suggested that the octamer may be organized into two heterotopic tetramers about a dyad axis of symmetry (28a). Histone H1 is believed to lie on internucleosomal DNA and/or along the nucleosomal surface (15, 18, 29, 30).

Several groups have demonstrated that the nucleosome repeat is composed of a nuclease resistant core of about 160 to 130 base pairs, and a nuclease-sensitive spacer (10, 11, 15, 30, 31). Recent evidence indicates that variation exists in DNA repeat lengths among different eukaryotes and cell types (32-37). This has been attributed to differences in spacer DNA lengths external to an evolutionally conserved 140 base pair core (32-35, 37). However, it is conceivable that more than one type of core may exist within the chromatin of a single cell nucleus. During the course of nuclease digestion several different core DNA lengths are observed simultaneously; no single DNA fragment stoichiometrically accumulates and 11 discrete DNA products are found at the limit of digestion (10-12, 15). It is possible, therefore, that certain of these components may arise from different classes of polynucleosomes. Indeed, subunits

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within transcribed chromatin have an altered conformation (38, 39), certain histone classes contain subfractions with different primary structures (40, 41), histones are known to undergo a variety of post-translational modifications (41), and non-histone proteins may contribute to nucleosome variability (12, 39, 42, 43).

In order to investigate the question of nucleosome heterogeneity, we have developed techniques capable of mapping simultaneously the precursor-product relationships, DNA lengths, and protein compositions present in a complex population of nucleosome oligomers. Application of these procedures to a study of bovine thymus chromatin has revealed five monomer nucleoprotein complexes which arise from at least two different parent DNA repeat lengths. Certain aspects of the precursor-product relationships and protein compositions of these monomers have been defined.

EXPERIMENTAL PROCEDURE

Materials

Electrophoresis grade agarose and Coomasie brilliant blue R-250 were obtained from Bio-Rad Laboratories, Richmond, Calif. "Stains-all" (No. 2718) and acrylamide were purchased from Eastman KODAK Co., Rochester, N. Y. The acrylamide was recrystallized from chloroform. Ethidium bromide was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisc. SDS was acquired from Alcohol, Inc., Baltimore, Md. Phenylmethylsulfonyl fluoride and Acridine black were purchased from Sigma Chemical Co., St. Louis, Mo.

Micrococcal nuclease was purchased from Worthington Biochemical Corp., Freehold, N. J. Hind III restriction endonuclease was obtained from Miles Laboratories, Inc., Kankakee, Ill. Restriction endonuclease 

Hae III was purchased from New England Bio Labs, Beverly, Mass. Nuclease-free pronase was obtained from Calbiochem, La Jolla, Calif. Proteinase K was purchased from EM Laboratories, Inc., Elmsford, N. Y. Bacteriophage PM2 DNA was the generous gift of Dr. Donald Gray, University of Texas at Dallas. Hind III restriction endonuclease fragments of SV40 DNA (strain 777) were the generous gift of Dr. Maxine Singer, National Institutes of Health. SV40 DNA (strain 779) was the generous gift of Dr. Bernard Hay, Swiss Institute of Experimental Cancer Research.

Methods

Isolation of Nuclei—All procedures were carried out at 0-4°C. Nuclei were prepared from calf thymus according to the method of Blobel and Potter (44). Fresh calf thymus was obtained from a local slaughterhouse and transported to the laboratory in Solution A (300 mM sucrose, 50 mM trithionanolamine, 25 mM KCl, 5 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride. pH 6.5 at 4°C). In all where phenylmethylsulfonyl fluoride was used, solutions were prepared fresh in isopropanol alcohol and added to buffer with rapid mixing immediately before use. Membrane and connective tissue were removed and 25 g were minced and brought to 200 ml with Solution A. The material was homogenized at 40 V for 5 min using a Waring Blender with an antifoam device. After filtration through two layers of cheesecloth, crude nuclei were pelleted at 200 x g for 10 min, resuspended in 40 ml of Solution A, and reserumized, 2.5 volumes of Solution B (2.5 M sucrose, 50 mM trithionanolamine, 25 mM KCl, 5 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride, pH 6.5 at 4°C) were added. The solution was centrifuged for 90 min at 115,000 x g through a 20% (v/v) cushion of Solution B. Resutling purified nuclei have a chemical composition of 1.0:1.1:1.43 = 0.12:0.049:0.002 (DNA:proteinfoRNA, means ± standard deviations, n = 6).

Enzymatic Preparation of Chromatin—Chromatin was prepared by a modification of a published technique (45). Freshly isolated nuclei were washed twice at 4°C by resuspension in Solution C (300 mM sucrose, 50 mM trithionanolamine, 25 mM KCl, 4 mM MgCl2, 1 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, pH 7.0 at 4°C) followed by centrifugation at 800 x g for 10 min. Nuclei were resuspended in Solution C to yield 5 mg/ml of DNA and preincubated 5 min at 37°C and micrococcal nuclease was added with rapid mixing to yield between 100 and 400 units/ml, depending on the experiment (stock solutions of the enzyme were stored at -20°C in Solution C minus phenylmethylsulfonyl fluoride at 20,000 units/ml. Following incubation from 30 to 15 min, depending on the experiment, samples were cooled rapidly to 0°C, portions were removed for acid solubility determinations (see below), and the remaining nuclei were pelleted by centrifugation at 800 x g for 10 min at 4°C. Supernatants were removed and 25 g were minced and brought to 200 ml with Solution A. The resulting mixture was centrifuged at 10,000 x g for 10 min at 4°C. The chromatin supernatant was used immediately for subsequent experiments, and consisted of 84 to 96% of the total, acid-insoluble nuclear DNA. Greater than 97% of the total acid-insoluble nuclear DNA was pelleted with the nuclei following nucleosome treatment. Furthermore, incubation of nuclei in the absence of 

Restriction Endonuclease Digestions—Purified SV40 DNA (5 μg) was treated with 2 units of Hind III in 50 μl of 50 mM NaCl, 6 mM Tris, 0.1 M MgCl2, 0.01% bovine serum albumin at pH 7.4 for 2 h at 37°C. Purified PM2 DNA (5 μg) was treated with 2 units of Hae III in 50 μl of 6 mM NaCl, 6 mM Tris, 6 mM MgCl2, 6 mM 2-mercaptoethanol, pH 7.4 for 2 h at 37°C. Partial digestion of DNA—DNA was purified after incubation of samples with 100 μg/ml of proteinase K in 1 mM NaCl, 10 mM EDTA, pH 8.0 at 37°C for 1 h (46), followed by extraction with re distilled phenol (equilibrated with 10 mM Tris, pH 8.0), and by three extractions with chloroformisomc alcohol (24:1, v/v). The aqueous phase was precipitated with 2 volumes of 95% ethanol at -20°C; the yield was greater than 90%.

Chemical Assays—DNA content was measured by absorbance at 260 nm in 1 N NaOH (27 liters per mg); samples containing high molecular weight DNA were dispersed by homogenization. Acid-soluble DNA was determined by measurement of the absorbance at 260 nm of material soluble in cold 0.5 M H2SO4, above that present in control nuclei (e = 33.2 liters per mg). RNA and DNA content were measured in ethanol-washed, 0.1 N H2SO4 precipitates after hydrolysis with 0.5 N KOH at 37°C and 0.5 N HClO4 at 100°C, respectively (41). Total nuclear protein was estimated by first precipitating samples with cold 25% trichloroacetic acid, washing with 95% ethanol at 4°C, dissolving residues in 0.5 N NaOH, and performing protein assays (48) using bovine serum albumin as a standard (εmax = 0.6 liters per g).

Electrophoresis of DNA—Vertical slab gel electrophoresis (20 × 17 × 0.3 cm) was performed using apparatuses patterned after Studier (49). Agarose (2%) gels were made and run in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8.5 (30). Acrylamide gels (4%, 20:1, acrylamide:N,N'-methylenebisacrylamide) used 0.1% SDS, 36 mM Tris, 90 mM sodium phosphate, 10 mM EDTA, pH 7.8, as the running buffer. Samples (0.5 μg of nuclear DNA standards) were loaded in 0.25× gel buffer, 10% sucrose. For 4% acrylamide, 0.1% SDS gels, samples contained in addition 1% SDS. Bromphenol blue (25%) was used as tracking dye. All DNA gels were run at room temperature.

For two-dimensional electrophoresis to display double-stranded DNA, first dimension tube gels (12 × 0.3 cm) were soaked for 1 h at 24°C in 1.0% SDS, 36 mM Tris, 30 mM sodium phosphate, 10 mM EDTA, pH 7.8, laid horizontally across the top of preformed 4% gel, and run at 20 mA for 1.5 hr. The stacking gel was used as 95% ethanol at 4°C. RNA and DNA content were measured in ethanol-washed, 0.1 N H2SO4 precipitates after hydrolysis with 0.5 N KOH at 37°C and 0.5 N HClO4 at 100°C, respectively. Total nuclear protein was estimated by first precipitating samples with cold 25% trichloroacetic acid, washing with 95% ethanol at 4°C, dissolving residues in 0.5 N NaOH, and performing protein assays (48) using bovine serum albumin as a standard (εmax = 0.6 liters per g).

Gels were stained either with Stains-all as described (46) and scanned at 550 nm using a 20-mm Gilford 2820 gel scanner with a 0.05-mm-wide slit, or with 1 μg/ml of ethidium bromide in water (5 μg/ml for single-stranded DNA) for 1 h and photographed using a short- wave ultraviolet lightbox (Ultraviolet Products) with Eastman Kodak X film using a 12Y or 15Y filter. In cases where SDS had been present
it was necessary to remove this material prior to staining by soaking gels in 50% methanol for 4 h with three changes (gels contract considerably but upon exposure to water for 1 h they largely regain their original dimensions).

DNA Length Measurements—Hae III restriction endonuclease fragments of PM2 DNA were used as markers for DNA length estimates by constructing standard curves of mobility versus log DNA lengths of chromatin fragments from the same tissue are a result of inaccuracies of DNA lengths assigned to standards and to differences in the extents of digestion. Gel Electrophoresis of Chromatin—Unless otherwise stated, enzymatically prepared chromatin was separated by electrophoresis at 4°C using 2.5% acrylamide, 0.5% agarose (acrylamide:N,N'-methylenebisacrylamide in 0.5x TBE buffer (12 x 0.1 cm) or slab gels (20 x 17 x 0.3 cm) with 6 mA Tris, 3.6 mM sodium acetate, 0.22 mM EDTA, pH 8.0, as the buffer system with recirculation. A level interface on tube gels was formed by use of dialysis tubing to seal gel tube bottoms during polymerization; after polymerization gels were inverted. Samples were loaded in 10% sucrose, 0.1% SDS, 0.25% bromphenol blue. Unless otherwise stated, sample loads for two-dimensional runs ranged from 10 to 20 μg of DNA, and 50 μg for one-dimensional slabs. Electrophoresis was 1.25 h for tubes at 250 V and 2.25 h for slabs at 20 mA. Material quantitatively enters; no precipitation of DNA occurs during polymerization; the nucleases were reactivated by soaking gels in 1 ml of micrococcal nuclease (100 units) in electrophoresis buffer (32). For purposes of DNA separation of mouse Ehrlich ascites chromatin, it is in part related to the chromatin source; two dimers species are resolved clearly when cultured mouse cell chromatin is separated under our conditions.

Second Dimension Mapping of DNA Composition—A second dimension of electrophoresis in the presence of SDS was employed to systematically map the DNA fragments of the separated nucleoprotein complexes described above. Fig. 2 shows a control experiment which indicates that DNA and protein migrate independently under the conditions used; the mobilities of DNA fragments of chromatin samples of two different digests are identical before and after rigorous purification of DNA. Histone H1 migrates at the leading edge of nucleosome DNA fragments in early digests, or between monomer bands of late digests. This causes quenching of DNA staining by Stains-all or ethidium bromide, but does not affect interpretation of the results below.

Fig. 3 summarizes second dimension maps which display the DNA fragments of electrophoretically separated chromatin; nucleoprotein electrophoresis is left to right; DNA separation is top to bottom. In order to calibrate the DNA lengths of the fragments thus resolved, one-dimensional mobility comparisons with restriction fragments of PM2 DNA were performed (Fig. 3, upper left panel; Fig. 4), and each second dimension gel includes at its side a one-dimensional standard of the same chromatin sample. The results of the one-dimensional calibration agree with the findings of others (10, 11, 15, 32, 53) that during the course of digestion several monomer and submonomer bands arise and become internally degraded. Slab gels were stained with Stains-all and scanned as described above.

Redigestion of Electrophoretically Separated Chromatin—First dimension tube gels were prepared and run as outlined above, with the following exceptions. Micrococcal nuclease or DNase I were added to gel solutions at 55°C (3.5% acrylamide, 0.5% agarose for DNAse I) just prior to pouring to yield 100 units/ml final concentrations. Chromatin samples (40 or 80 μg DNA for micrococcal nuclease and DNase I experiments, respectively) were separated electrophoretically with 100 units/ml of micrococcal nuclease or DNase I dissolved in the lower or upper electrode buffers, respectively. Dialysis bags containing 1 ml of micrococcal nuclease (100 units) in electrophoresis buffer were attached to the lower end of gel tubes to conserve enzyme. After electrophoresis, the nuclease was reactivated by soaking gels in 1 mM triethanolamine, pH 7.4, containing either 1 mM CaCl₂ or 1 mM MgCl₂, at 37°C for periods up to 30 min. For micrococcal nuclease digestions, reactions were terminated by soaking gels for 30 to 60 min in 1.0% SDS, 36 mM Tris, 30 mM sodium phosphate, 10 mM EDTA, pH 7.8; electrophoresis in the second dimension was performed as described above. For DNase I digestions, reactions were terminated by soaking gels for 1 h in 1.0% SDS, 40 mM Tris, 14.5 mM sodium borate, 2.5 mM EDTA, pH 8.3, followed by soaking gels for 1 h in the same solution plus 10 mM urea, and heating to 85°C for 5 min. (Since gels lose their agarose matrix under these conditions, 1% SDS, 1 μM NaOH may be used as a substitute for the heating step.) Electrophoresis in the second dimension was performed as described above.
appear as skewed spots, while monomer III appears as a cross-hatched line. In early digests there is an overlap in the DNA sizes of monomers I, II, and III (Fig. 3a). During the course of digestion there is a gradual reduction in monomer DNA sizes. The cross-hatched line of monomer III turns to eventually become a band. Concomitantly, the DNA size of monomer III changes from a range of about 210 to 160 base pairs to approximately 160 base pairs and monomer II becomes masked (compare Fig. 3, B and F; Fig. 4). Nuclease processing of monomer II results in DNA fragments of 160 base pairs from material initially ranging between 185 to 160 base pairs in length (Fig. 3f). Monomer I has a DNA size range of about 175 to 140 base pairs, and becomes trimmed to approximately 140 base pairs in late digests (Figs. 3 and 4). Therefore, the core DNA lengths (15) of monomers I, II, and III are 140, 160, and 160 base pairs, respectively. It is difficult to predict the degree of homogeneity of these monomers with respect to protein composition or particle conformation, or both, from the shapes of their second dimension DNA patterns. DNA size differences for a particle with a homogeneous protein content would be expected to create opposing effects on its electrophoretic mobility; size reduction being accompanied by a reduced net charge.

In contrast to monomer DNA profiles, dimer nucleosomes and higher multimers are separated in the first dimension principally on the basis of DNA size; after electrophoresis in the second dimension a diagonal line exists, as opposed to spots or bands for the DNA components of each oligomer class (Fig. 3). With regard to dimer DNA patterns, a spot appears to move through a less intense diagonal line during the course of digestion; at early periods the spot appears at the top of the diagonal, while at later periods at the bottom. This shows clearly the change in the mass distribution of different DNA lengths for dimer, which is summarized in quantitative terms in Fig. 4.

The slight streaking of oligomers depicted in Fig. 3E was of interest since it occurred only among the longest DNA lengths present within each multimeric class. Furthermore, the appearance of these larger multimers correlated well temporally with the appearance of a faint spot toward the upper left of the cross-hatched line of monomer III DNA (Fig. 3E). These observations suggested that a larger repeat may exist which was more resistant to nuclease and was a precursor to the faint spot neighboring monomer III DNA. Evidence strongly supporting this notion was obtained using different electrophoretic conditions. As shown in Fig. 6, two additional monomers can be resolved in late digests, termed IV and V, with DNA length ranges of 185 to 205 and 190 to 220 base pairs, respectively. These apparently arise from the larger oligomers depicted diagrammatically in Fig. 6B, which yield a repeat length of 203 ± 9 base pairs as judged by averaging the
Fig. 3. Second dimension mapping of the DNA composition of electrophoretically separated chromatin. The upper left panel shows the separation of chromatin DNA fragments from nuclear digestions of: A: 2%; B, 5%; C, 9%; D, 12%; E, 17%; and F, 21%. Electrophoresis (top to bottom) was performed using a 4% acrylamide, 0.1% SDS slab gel. Hae III restriction endonuclease fragments of PM2 DNA are included as standards. The other panels show two-dimensional separations of the same chromatin samples shown in the upper left panel, where nucleoprotein electrophoresis was the first dimension (left to right), and DNA electrophoresis was the second dimension (top to bottom). A one-dimensional standard of the same chromatin sample has been included at the side of each two-dimensional gel as an internal standard. Ethidium bromide was used for staining.

Fig. 4. Changes in DNA lengths of bovine thymus chromatin fragments during the course of nuclease digestion. Data from Fig. 3, upper left panel, were used for size estimates. The dashed lines indicate diffuse doublet bands of 124, 120 and 104, 100 base pairs which appear in trace amounts later in digestion.

These presumably account for less than 1% of the total chromatin DNA. Therefore, the nucleosomal repeats of bovine thymus chromatin range from 203 to 142 base pairs, with the great majority of the nucleosomal DNA centering about a unit size of 191 base pairs.

Quantitation of Poly- and Mononucleosomal DNA – Under the digestion conditions used, bovine thymus mononucleosomes accumulate with time and show little internal breakdown prior to the depletion of multimers (Fig. 3). Thus, the amount of chromatin organized in nucleosomal structures can be evaluated from the extent of digestion required to quantitatively convert the global repeat of 191 ± 8 base pairs to equal masses of 160 and 140 base pair fragments. As shown in Fig. 3F, essentially complete conversion of chromatin to these fragments occurred after 21% digestion, closely agreeing with a theoretical value of 41/191 = 21.5% for the total participation of nuclear DNA in a homogeneous repeat. Although the presence of other DNA repeat lengths complicates this comparison, it appears that only a few per cent, at most, of bovine thymus chromatin can exist in structures other than polynucleosomes. This supposition has been strengthened by quantitative electron microscopy studies.

The relative proportions of the various mononucleosomes are not constant during the course of digestion. In particular, monomers IV and V appear transiently; they are not visible in early or late digests (Fig. 3) and, under optimum conditions, account for only a few per cent of the total monomer population (Fig. 6). On the other hand, monomer I accumulates during digestion. Quantitation by an analysis of areas of one-dimensional gels reveals that monomer I comprises between 28 and 33% of the total monomer population upon proceeding from 2 to 14% digestion (Fig. 1). In agreement with Varshavsky et al. (29), monomer I accounts for a greater percentage in later digests, suggesting that larger monomer(s) may be precursors to this component (Fig. 3F). Estimates of the proportion of monomer II from two-dimensional gels suggest that

**Differences between successive multiples (32).** This value significantly differs from 188 ± 3 base pairs, the unit size estimated for the major polynucleosomal class resolved in this experiment (Fig. 6C).

The finding that about 5% of bovine thymus chromatin consists of a different repeat which is more resistant to nuclease prompted us to explore the DNA profiles of yet more extensive digests. As shown in Fig. 7B, a high DNA load of a near limit nuclear digest reveals two additional minor repeats of 170 ± 8 and 142 ± 3 base pairs, estimated by averaging the differences between successive multiples of trimers through nanomer and monomer through tetramer, respectively (32).
this component may comprise 10 to 20% of the total monomer population (Fig. 3).

"Chromatin Fingerprinting" — It seems clear that monomers IV and V exist in chromatin in a tandem arrangement, independent of monomers I, II, and III. The larger monomers appear during digestion only when larger multimers are visible, ruling out a general interspersed organization (Figs. 3E and 6). The question then arises: Do monomers I, II, and III exist in chromatin as independent structures, perhaps tandemly, or do they originate from a structurally and compositionally identical monomer by asymmetric nuclease cleavages or by nuclease processing (or both)? To approach these issues, a chromatin fingerprinting technique has been developed (1). After electrophoretic separation of nuclease complexes, samples are digested further prior to second dimension DNA electrophoresis. This is accomplished by incorporating reversibly inactivated micrococcal nuclease or DNase I within the first dimension gel matrix and running buffer; the enzymes are reactivated after electrophoresis by exposing gels to Ca²⁺ or Mg²⁺ ions. The technique thus allows precursor-product relationships between all components to be monitored simultaneously.

The results of three independent time course experiments which employed redigestion with micrococcal nuclease reveal a complexity which is highly reproducible (Fig. 8, A to D, E to H, and I to L). The intact patterns of controls not exposed to Ca²⁺ ion indicate that during electrophoresis of chromatin the nuclease is inactive and does not alter the details of separation (Fig. 8, A, E, and I). Upon nuclease reactivation, it is clear that each multimer, n, is a precursor to the next smaller multimer, n - 1 (Fig. 8, B, H, and K), a point supported earlier by redigestion experiments on pooled oligomers (15, 57). It is noteworthy that the 160 base pair cores of monomers II and III are first processed to 140 base pairs and then to two smaller doublets of 124, 120 and 104, 100 base pairs, the same submonomer fragments observed upon redigestion of monomer I (Fig. 8, K and L). We conclude that monomers II and III are precursors to monomer I. Since the above submonomer DNA lengths agree closely with published values for chromatin and unfractionated mononucleosome limit digest bands (10-12, 15, 58), the complexity of submonomer digestion products need not represent structural heterogeneity beyond that contained in monomer I per se. This conclusion is strengthened further by the fact that the three monomers give rise to identical submonomer single-stranded DNA patterns upon DNase I redigestion (Fig. 9), patterns shown earlier to be characteristic of DNase I action on chromatin (59) and unfractionated mononucleosomes (15).

Do classes of polynucleosomes exist in the global repeat which are composed only of monomer I? The finding that monomers II and III are precursors to monomer I complicates a solution to this question, particularly if chromatin consisting of monomer I per se is more resistant to nuclease digestion. However, if the major repeat is composed of a distribution of polynucleosomal classes with slightly different repeat lengths, then chromatin fingerprint patterns have great discriminatory potential. This follows from the fact that dinucleosomes and larger multimers are fractionated electrophoretically principally on the basis of DNA length (Figs. 3, 6, and 8). Hence, upon redigestion, different geometric patterns of product DNA fragments would be expected if clustered microheterogeneity exists. Indeed, Fig. 10 shows that different tetram mer product DNA patterns can be predicted from basic principles for molecules originally exhibiting length heterogeneity due to exonuclease trimming, spacer length, core organization, or both spacer and core size.

Reinspection of the data of Fig. 8 with reference to the models shown in Fig. 10 reveals several interesting points.
During the course of redigestion, nearly all multimers are first converted to products showing 160 base pair barriers which then decay to 140 base pair fragments (Fig. 8). Therefore, it is clear that the great majority of polynucleosomes are organized with cores of 160 base pairs. The fact that a diagonal line is maintained upon conversion of tetramers to dimers (Fig. 8, B, H, and K) implies that there is clustered microheterogeneity in the global repeat due to spacer variability about 160 base pair cores (Fig. 10, model II). If histone H1 determines the spacer size as suggested by Noll (32), then such microheterogeneity might be due to different tandem arrays of the various H1 species of bovine thymus (41). Since both monomers II and
III consist of 160 base pair cores, further questions are raised concerning their possible organization in chromatin which remain to be answered.

With regard to monomer I, direct conversion to 140 base pairs, without a pause at 160 base pairs, is seen upon redigestion of small dimers (Fig. 8, B, F, and G). This may be due to end degradation during nuclear digestion which may predispose a direct 140 base pair product upon redigestion (Fig. 10, model I). Alternatively, a minor class of polynucleosomes may be composed of monomer I (Fig. 10, model III). That this species may exist tandemly in a minor portion of chromatin is supported by the 142 ± 3 base pair repeat described above (Fig. 7).

Other redigestion experiments offer direct support that the larger 203 ± 9 base pair repeat is a precursor to monomers IV and V; conversion of large trimers to large dimers and monomers has been observed (not shown). However, due to the low concentrations of monomers IV and V, it has not been possible to map the precursor-product relationships or core structures of these components.

Second Dimension Mapping of Protein Composition—A number of investigators have shown that 140 base pair monomers lack histone H1, but contain approximately equal proportions of the other histone species (15, 28, 29, 52). However, whether lysine-rich species are present in monomers with longer DNA lengths is unclear; studies on HeLa and erythrocyte mononucleosomes have revealed only traces of H1 and H5 (15, 28, 52), while larger sized mouse monomers have been reported to be rich in H1 species (29). In the present study, establishing the location of H1 species among bovine nucleosome monomers was particularly attractive in view of the knowledge accrued on precursor-product relationships.

To display the chromatin proteins of electrophoretically separated bovine chromatin, a second dimension of electrophoresis using 18% acrylamide, 0.1% SDS slabs was employed (54).
a lower percentage of acrylamide. After staining for both DNA and protein, the protein composition of different monomers could be established with certainty due to the close proximity of the component DNA species. As shown in Fig. 11B, monomer II lacks H1, while monomers IV and V appear to contain a complete histone complement.

**DISCUSSION**

Table I summarizes the properties of the five mononucleosomes of bovine thymus chromatin which have been identified in the present study. It is of interest that during the course of nuclease digestion there is partial overlap in the DNA lengths between different monomer classes. In particular, monomers I, II, and III share common lengths; monomers III, IV, and V show similar overlaps. Thus, parameters in addition to DNA size contribute to mononucleosomal fractionation by electrophoresis. These presumably include protein composition, charge, and conformation. The electrophoretic complexity of mononucleosomes cannot be explained solely on the basis of histone H1 content, since both monomers I and II lack these species. Furthermore, Ollins et al. (52) have shown that electrophoretic fractionation of erythrocyte mononucleosomes is not entirely due to lysine-rich histone content. Thus, whether the five classes of monomers described above each consist of a histone octamer (17) composed of two copies of each of the four smaller histones remains to be established.

A paucity of monomer I exists in bovine thymus chromatin; the great majority of polynucleosomes are organized with cores of 160 base pairs. This value, as opposed to 140 base pairs, agrees with the core size predicted for rat liver chromatin by Simpson and Whitlock (31). Although we have established that monomer I accumulates during digestion because it is a processing product of monomers II and III, it is not clear whether monomer III is processed to monomer II or if monomer II exists independently in chromatin.

Histone H1 binding domains on spacer DNA have been suggested previously (15, 29, 31). The results of the present study imply that an additional binding site(s) exists on or near the nucleosomal surface. H1 remains bound to monomer III even after nuclease processing to 160 base pairs. The conversion of this form of monomer III to monomer I is accompanied by digestion of 20 base pairs and release of H1. Recent direct findings of Varshavsky et al. (29) support the above results; however, a discrepancy in DNA lengths exists which presumably reflects differences in size calibrations. Whether single H1 molecules occupy 20 base pairs on one or two terminal sites or become released indirectly are matters for further study. The recent finding of Olins et al. (52) demonstrating equal molar ratios of the five classes of histones in erythrocyte nuclei further complicates this issue.

Since the major polynucleosomal repeat of bovine thymus chromatin is 191 ± 8 base pairs, an average length of about 30 base pairs can be surmised for the spacer which connects 160 base pair cores. Clustered variation in spacer lengths is suggested by the maintenance of diagonal lines in chromatin fingerprints (Fig. 8, B, F, and K; Fig. 10, model II; Ref. 1). Indeed, the DNA length ranges of multimers are sufficiently broad not to preclude the existence of a distribution of repeating units, each differing by several base pairs from the mean value. Variation in the global repeat length may be due to different histone H1 subfractions, if lysine-rich histones are responsible for determining the distance between adjacent cores as suggested earlier (32). Clustering of spacers of similar lengths could be due to specialized organizations of H1 subfractions; chemical cross-linking studies support the existence of homopolymers of lysine rich histone subfractions in chromatin (60).

In addition to the global nucleosomal repeat discussed above, at least three other minor classes with different repeat lengths exist in bovine thymus chromatin. These findings raise a number of questions with regard to the origins and functions of varied arrangements of nucleosomes. Different repeats may arise from the various cell types of thymus tissue. Recently, nucleosomal repeat lengths have been shown to differ between chicken tissues (61). Conversely, different repeats may exist within single cells and may possibly represent various functional states of the genome. It is conceivable that certain classes may even be organized with subsets of the total nuclear DNA sequences. These possibilities are currently under investigation.

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**Note Added in Proof**—Recently, we have found that monomers I, II, III, and IV-V are present in the chromatin of embryonic bovine trachea cells (ATCC CCL44). This finding demonstrates that unique mononucleosomes which arise from different parent DNA repeat lengths exist within a single cell type. The DNA sizes of these mononucleosomes are similar to those of bovine thymus chromatin, except that monomer II is trimmed to 140 base pairs. This observation offers further support to the proposal that differences in protein composition or particle conformation, or both, exist between monomers I and II.

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