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Nuclease Action on Chromatin: Evidence for Discrete, Repeated Nucleoprotein Units Along Chromatin Fibrils

(non-random digestion/11S subunits/electron microscopy/DNase II/calf thymus)

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ABSTRACT **The time course of the fragmentation of calf thymus chromatin by DNase II (deoxyribonucleate 3′-oligonucleotidohydrolase, EC 3.1.4.6) has been examined by sedimentation of chromatin digests through linear (5–20%) sucrose gradients. The action of the nuclease is decidedly nonrandom, ultimately producing roughly equal amounts of acid-soluble oligonucleotides and 11S nucleoprotein particles. The 11S particles contain double-stranded DNA that is approximately 400 Å or 120 base-pairs long, as measured by electron microscopic examination of deproteinized samples, and is maintained in a compact conformation within the intact particles. In addition, 15S nucleoprotein particles containing predominantly 800-Å lengths of DNA have been isolated from less extensively digested chromatin. Evidence is presented which indicates that the 11S particles are fundamental structural units that are arranged in tandem along certain regions of chromatin fibrils. Preliminary experiments with different nucleases and with chromatin from different mammalian species indicate that these results are a natural consequence of the arrangement of DNA and proteins in mammalian chromatin and are not peculiar to the system described in detail.**

Chromatin isolated from higher organisms is a fibrous nucleoprotein consisting of DNA closely associated with histones and other proteins that regulate gene activity and maintain DNA in a compact state within the nucleus. Despite extensive efforts, descriptions of the distribution of proteins along DNA and of the folding of nucleoprotein fibrils in chromatin have remained uncertain. Recently a number of lines of evidence have suggested that DNA is arranged in small, repeating nucleoprotein units along chromatin fibrils. Olins and Olins (1) have observed closely spaced, roughly spherical particles with diameters of 70 Å in electron micrographs of chromatin strands streaming from ruptured nuclei. Rill and Van Holde (2) and Sahasrabuddhe and Van Holde (3) have isolated up to 50% of the total chromatin DNA in the form of small (180,000 daltons), compact (frictional ratio $f/f_0 = 1.1$), nucleoprotein fragments that are resistant to the action of micrococcal nuclease (nucleate 3′-oligonucleotidohydrolase, EC 3.1.4.7). Digestion of rat liver chromatin by an endogenous nuclease was found to occur nonrandomly, producing discrete classes of DNA fragments that are multiples of a basic size unit (4). Additional findings that arginine-rich histones F2A1 and F3 form a stable tetrameric complex (5) and appear to be clustered along DNA in chromatin (6) have been suggested as indirect evidence for a repeating histone–DNA unit.

Examination of the action of nucleases on chromatin has provided strong additional evidence of a subunit structure. We have analyzed in considerable detail the fragmentation of chromatin into small nucleoproteins with time upon treatment with low levels of DNase II (deoxyribonucleate 3′-oligonucleotidohydrolase, EC 3.1.4.6) and other nucleases. The breakdown of chromatin is decidedly nonrandom, leading ultimately to an approximately equal mixture (in terms of DNA phosphate) of acid-soluble oligonucleotides and 11S nucleoprotein particles containing double-stranded DNA that is 400-Å long and is tightly folded in the intact nucleoprotein. Apparent multiples of the 11S particles containing predominantly 800-Å, 1200-Å, and 1600-Å lengths of DNA are observed in less extensively digested samples.

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MATERIALS AND METHODS

Chromatin Preparation. Calf thymus chromatin was prepared from previously quick-frozen thymus essentially by the method of Bonner *et al.* (7). Sodium bisulfite was incorporated into the solutions to prevent proteolysis (8). Crude chromatin was further purified by sedimentation through 1.7 M sucrose. All chromatin preparations contained the full complement of histones as judged by electrophoresis on polyacrylamide gels.

DNase II Digestion of Chromatin. Nuclease digestions were performed within 24 hr after chromatin preparation. Chromatin pellets in sucrose were washed three times with digestion buffer [5 mM $NaHSO_3$, 0.1 mM Na_2EDTA, adjusted to pH 7.0 with solid tris(hydroxymethyl)aminomethane], gently suspended in buffer with a Teflon-in-glass hand homogenizer, and diluted to give a final DNA concentration of approximately 200 μg/ml. Samples were equilibrated for 30 min at 37°, then inoculated with 2 μg of DNase II per 100 μg of DNA. Digestion was inhibited at the times indicated below by addition of an equal volume of ice-cold 50 mM Tris, 5 mM $NaHSO_3$, 0.1 mM Na_2EDTA. (Since the pH optimum of DNase II is approximately pH 5, the enzyme activity is low at pH 7 and is additionally reduced by adding Tris to give a final pH of 8.4–8.5.) A portion of each digest, later to be used for chemical analysis, was quick-frozen in a dry ice–acetone bath and stored at −20°. The remaining sample was immediately layered onto cold sucrose gradients.

Velocity Sedimentation in Sucrose Gradients. The size distribution of nucleoprotein fragments produced by various degrees of digestion was analyzed by centrifugation on linear 5–20% sucrose gradients containing 25 mM Tris, 5 mM $NaHSO_3$, 0.1 mM Na_2EDTA, pH 8.4. Centrifugation was done at 40,500 rpm in an International SB-283 rotor maintained at 5°. Gradient absorbance profiles were measured

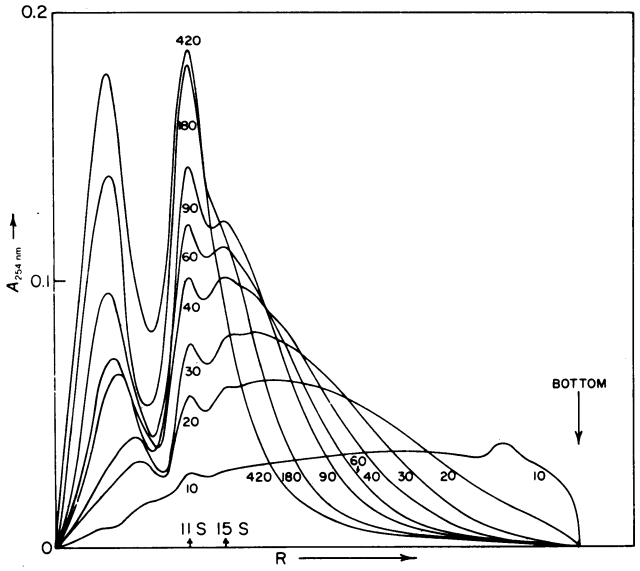

FIG. 1. Sucrose density gradient (5–20%) sedimentation profiles of calf thymus chromatin digested with DNase II for the number of minutes indicated. Digestion conditions are given in the *text*. Centrifugation was for 6 hr at 40,500 rpm in an International SB-283 rotor maintained at 5°.

with an Isco density gradient fractionator and model UA-5 absorbance monitor. Radial positions in the gradient patterns were converted to approximate $s_{20,w}$ values with a simple computer program which assumes that the viscosity and density corrections are linear with distance over short regions of the tube.

Electron Microscopy of Isolated DNA. To destroy residual nuclease activity the effluent from the gradient fractionator was dripped into enough concentrated sodium dodecyl sulfate solution to give a final detergent concentration of 1% in each tube. Selected fractions were deproteinized with chloroform–isoamyl alcohol (24:1, v/v) and dialyzed at 4° against 10 mM Tris·HCl, 1 mM EDTA, pH 8.5. The DNA concentration in the final solutions was 10–15 μg/ml. A variation of the basic protein film technique was used to mount DNA for observation with the electronic microscope (9). The spreading solution contained 22 μl of DNA solution, 3 μl of cytochrome *c* (1 mg/ml), and 25 μl of 2.0 M ammonium acetate, pH 7.5. The hypophase was 0.25 M ammonium acetate, pH 7.5. The film was picked up on Parlodion-covered grinds, stained with 50 μM uranyl acetate dissolved in 90% ethanol, washed in isopentane, and rotary shadowed with platinum:palladium (80:20). Grids were observed and photographed with a Philips EM200 electron microscope. Electron micrographs were projected with a total magnification factor of 172,000× and tracings of the DNA molecules were made. The electron microscope was calibrated using a carbon replica of a 54,860 lines per inch (21,600 lines per cm) diffraction grating (Ladd Research Industries, Inc.). Calibrations were performed before and after photographing the specimen grids and agreed within 2–3% at each level of magnification.

Polyacrylamide Gel Electrophoresis of histones was performed on 15% gels containing urea as described by Panyim and Chalkley (10).

Measurement of Urea-Dissociable Protein. Digests and controls were made 6 M in urea by addition of 1.5 volumes of 10 M urea. Aliquots of each sample were then electrophoresed on polyacrylamide gels by the method employed for histones. Under these conditions the proteins migrate into the gel significantly, but are not resolved. Staining with amido black revealed one band, which was quantitated with the gel scanner attachment of the Isco absorbance monitor.

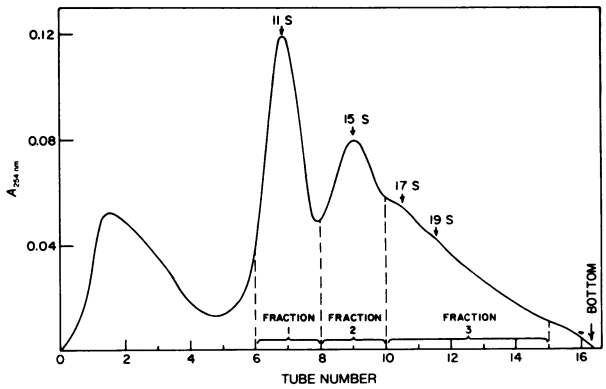

FIG. 2. Sucrose density gradient (5–20%) sedimentation profile of calf thymus chromatin digested for 40 min with DNase II. Centrifugation was for 10 hr at 40,500 rpm in an International SB-283 rotor maintained at 5°. Fractions pooled as indicated were used for DNA length measurements shown in Fig. 3.

Enzymes. Hog spleen DNase II, pancreatic DNase I (deoxyribonucleate 5′-oligonucleotidohydrolase, EC 3.1.4.5), and micrococcal nuclease were purchased from Worthington Biochemical Corp. and were of the highest specific activity available.

RESULTS

DNase II was chosen for our initial studies because it lacks a requirement for divalent cations, which aggregate chromatin and promote histone exchange (11). In addition, the early action of DNase II involves simultaneous cleavage of both DNA strands, hence chromatin fragments produced by DNase II may have fewer single-strand ends than those produced by other nucleases.

Under the conditions of our digestions the gel structure of chromatin is rapidly broken down. After incubation for only 2.5 min, the shortest times employed, 80% or more of the chromatin remains in the supernate after centrifugation at 10,000 × *g* for 15 min. Density gradient patterns of chromatin digested for 2.5–10 min (shown in Fig. 1) are extremely broad, suggesting nearly random cleavage. As the reaction proceeds further, the nonrandom nature of the digestion becomes evident as well-defined peaks appear in the gradients at the 11S and 15S positions and nonsedimenting material accumulates at the top of the gradient. Eventually a relatively stable state is reached in which approximately half of the sample is converted to nonsedimenting material and the remainder appears under the 11S peak (Fig. 1). Essentially no difference was observed between the profiles of the samples digested for 420 min and 300 min (not shown), and 37% of the DNA in both samples was reduced to acid-soluble oligonucleotides.

Gradient profiles of chromatin digested for intermediate times (40–60 min) contain two well-defined peaks at the 11S and 15S positions beyond the slow sedimenting material. In addition there appear to be two shoulders on the fast moving side of the 15S band, as is shown more clearly in Fig. 2, where

resolution has been improved by increasing the sedimentation time from 6 to 10 hr. Sedimentation coefficients (± mean deviation) for these four distinguishable components calculated from six different gradient patterns were found to be 11.3 ± 0.2 S, 14.6 ± 0.2 S, 17.3 ± 0.3 S, and 19.0 ± 0.3 S, respectively. (The absolute error in these measurements may be significantly larger than the precision indicated because of uncertainties in the deceleration time, meniscus position, etc.)

Measurements of the lengths of DNA pieces isolated from fractions 1, 2, and 3 shown in Fig. 2 suggest that the three fast-moving components are simple multiples of the 11S component. Histograms of the lengths of double-stranded DNA recovered from the fractions are given in Fig. 3C–E. The mean length of the DNA from fraction 1, which contains predominantly the 11S band, is 400 Å (Fig. 3C). This corresponds to approximately 120 base pairs or 80,000 daltons of double-stranded B form DNA, neglecting slight stretching which may occur during grid preparation and errors in estimating end positions. The distribution of lengths is relatively narrow, with 66% of the total molecules measured ranging from 340 Å to 460 Å. Molecules of this size are readily observed. In addition, the fact that molecules as small as 214 Å were observed and measured demonstrates that the mean length of 400 Å is not at the resolution limits of our methods. DNA from fraction 2, which contains predominantly 15S material, has a mean length of 800 Å, twice that of the 11S band DNA. Fraction 3 contains a wide distribution of sedimenting species, but the corresponding histogram clearly shows maxima at 1200 Å and 1600 Å, demonstrating that the shoulders observed on the gradient pattern are not artifacts but represent true multiples of the 11S particles.

Length histograms of DNA isolated from unfractionated samples digested for 40 and 10 min in a separate experiment are shown for comparison in Fig. 3A and B, respectively. While periodicity is not notable in this whole 40-min digest, the limit products of the digestion are clearly 400-Å lengths of DNA. As is expected from the broad gradient profile of a 10-min digest, the corresponding histogram spans a wide range of lengths, but peaks are observed near the 800-Å and 1200-Å positions.

Measurements of the amounts of acid-soluble oligonucleotide produced after incubation for 5–6 hr under the normal digestion conditions averaged about 50% ± 10% for several experiments, suggesting that the remaining 50% of chromatin DNA is contained within the subunits. At present our data do not permit a more exact estimate of the subunit content, since we have not attempted to push the nuclease reaction to completion. In addition we have found that determination of the total effect of nucleases on chromatin is complicated by the action of proteases that contaminate commercial preparations of DNase II and, to a lesser extent, DNase I. Electrophoretic analysis of histones extracted from total digests shows that histone F1 is degraded to faster moving peptides after incubation for 20 min and is, therefore, not intact in the isolated 11S particles. This sensitivity of histone F1 to proteolysis has been noted previously (8) and is consistent with the generally accepted view that histone F1 is the least tightly bound of the histones and is responsible for crosslinking chromatin fibrils. For this reason also we feel that the loss of histone F1 during digestion does not decrease the significance of the observed fragmentation pattern. The remaining four histone classes appear to be relatively resistant to protease action. In a few

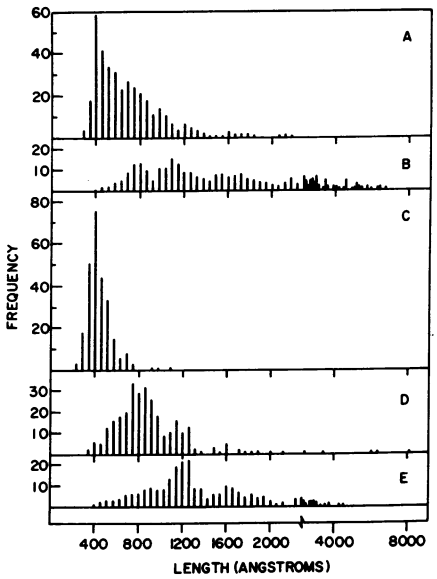

FIG. 3. Frequency diagram showing lengths of DNA pieces isolated from whole digests and individual sucrose density gradient fractions. (A) Calf thymus chromatin digested for 40 min, unfractionated. Number of molecules measured (N) = 384. (B) Calf thymus chromatin digested for 10 min, unfractionated, N = 303. (C) Fraction 1, containing predominantly 11S fragments, N = 258. (D) Fraction 2, containing predominantly 15S fragments, N = 303. (E) Fraction 3, containing 17S, 19S, and faster sedimenting fragments, N = 329.

cases we have observed a very slow loss of 11S material in the gradients after prolonged digestion. Histone gel patterns of such digests show a deficiency of histone F3, indicating that the degradation of this histone may cause a change in the nuclease susceptibility of the subunits.

Preliminary evidence suggests that the 11S subunits that we isolate from gradients are similar, if not identical, to the micrococcal nuclease-resistant chromatin fragments (termed PS particles) isolated by Rill and Van Holde (2) and Sahasrabuddhe and Van Holde (3). Both preparations have approximately the same sedimentation coefficient and contain the same length of DNA. The 11S subunits are insoluble in 0.15 M NaCl, as are the PS particles. Hydrodynamic studies by Van Holde and coworkers (2, 3) have shown that the PS particles have a compact, rather than an extended, conformation. For example, brief treatment of the PS particles with trypsin causes a 50% reduction in $s_{20,w}$ although only 20% of the protein is lost. Similarly, treatment of our 11S subunits with 6 M urea, which unfolds native chromatin without dissociating histones, caused a 50% reduction in $s_{20,w}$. Addition of sodium chloride to a final concentration of 2 M, which is sufficient to dissociate most of the histone from DNA, caused a decrease in $s_{20,w}$ of 56%.

We have been considerably concerned about the potential for artifact production during the nuclease digestion. Several established features of the nuclease action strongly indicate that the 400-Å spacing of nuclease-sensitive sites along DNA must exist in "native" chromatin.

Fragmentation patterns of chromatin as observed by sucrose density gradient centrifugation have proven to be extremely reproducible. Patterns virtually identical to those described have now been obtained in experiments with more than 15 preparations of calf thymus chromatin over a period of

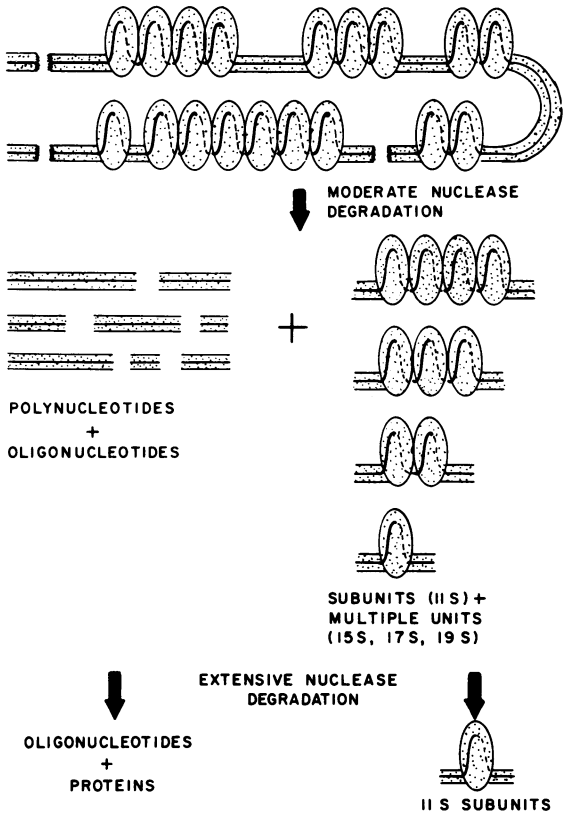

FIG. 4. Schematic representation of the substructure of chromatin fibrils and the action of nucleases on the fibrils. The solid line represents double-helical DNA. Proteins are represented by dotted areas. The model proposes that along certain regions of the fibril DNA is folded at regularly spaced intervals into discrete nucleoprotein units which are resistant to nuclease attack. Other regions of the nucleoprotein fibril have a more open structure which permits access to nuclease. No information is presently available on the relative lengths or distributions of folded and open regions. The supercoiling of DNA within the folded regions illustrates one of a number of possible DNA conformations. Nuclease action occurs predominantly in the open regions and at nuclease-sensitive sites between subunits, initially producing multiple units, which are eventually reduced to single units.

several months with different lots of calf thymus and DNase II. As will be detailed in a later report, we have also examined the action of DNase II on chromatin from calf liver, rat liver, and Chinese hamster ovary cells grown in culture, and the action of pancreatic DNase I and micrococcal nuclease on calf thymus chromatin. In all cases we have observed gradient patterns similar or identical to those reported above, indicating that our results are a consequence of a general structural feature of chromatin.

With the exception of histone F1 noted above, histones remain intact during most of the course of the digestion with DNase II. Recently we have found that micrococcal nuclease, which appears to lack protease activity, digests calf thymus chromatin into a mixture of 11S, 15S, and 17S components, plus a small amount of fast-sedimenting and slow sedimenting material, without degrading histone F1. Hence, proteolysis of histone F1 is not required for release of the subunits by nuclease.

Only 2–3% of the proteins on whole chromatin are dissociable in 6 M urea at low salt concentrations. Samples of chromatin digested with DNase II were made 6 M in urea and analyzed to determine the amount of DNA-free protein. Only 6–10% of the protein of chromatin digested for 5–6 hr was DNA-free, indicating that little protein is released into solution or loosely bound to undigested regions after nuclease digestion. While tight binding of migrant proteins could protect DNA from further digestion, it is difficult to imagine how the migration and binding of released proteins could create regularly spaced, nuclease-sensitive regions of DNA. In this regard it is most important to note that we observe the creation and build-up of the 11S and 15S components after relatively brief incubation with all of the enzymes studied, before there is significant production of acid-soluble oligonucleotides and urea-dissociable protein or degradation of histones. We conclude that neither histone proteolysis nor migration of proteins from digested regions is required for production of 11S units from chromatin. This does not rule out the possibility that these factors may influence analyses of the protein composition of the 11S units, particularly if they are isolated after prolonged digestion.

DISCUSSION

The fact that DNA in chromatin is maintained in a compact state is well documented (see for example, ref. 12). The data presented above suggest that part of this compaction is accomplished by the folding of approximately 400-Å long sections of DNA into a nucleoprotein unit that is resistant to nuclease attack. Our ability to identify integer multiples of this basic unit indicates that such units can be joined in tandem by short, nuclease-sensitive stretches of DNA. Since at present we are only able to isolate about 50% of the total DNA as 11S subunits, it is possible, and perhaps likely, that there are large "open" or unfolded regions of protein-associated DNA along chromatin fibrils that are readily accessible to nuclease. Such regions may correspond to the "template-active" portions of chromatin isolated by Marushige and Bonner (13) from chromatin treated briefly with DNase II. Alternatively they may arise in part from disruptions of the folded chromatin structure caused by handling.

A schematic representation of the action of nucleases on chromatin based on these considerations is shown in Fig. 4. The dispersion about the mean lengths observed in the DNA length histograms may logically arise from different spacings between the units and different lengths of the "tails" obtained upon cleavage of the open regions between subunits.

The model presented above is of the "beads on a string" type proposed previously by Olins and Olins (1) and Kornberg (5). An interesting feature of such a model is that it allows for compaction of the DNA in a regular manner while preserving a high degree of chain flexibility through the open segments joining compact units. Measurements of the rotational relaxation of chromatin fibers oriented by electric fields have shown that chromatin is, in fact, nearly as flexible as protein-free DNA (14). Olins and Olins (1) have proposed that the spheroid chromatin bodies observed in electron micrographs may contain equimolar amounts of the five histone classes bound to 80,000 daltons of double-stranded DNA. Kornberg (5) has suggested that the basic DNA repeat unit is 200 base pairs or about 680-Å long and consists of a compact region in which DNA is associated with a histone $(F2A1)_2(F3)_2$ tetramer and an extended region in which DNA is associated

with histones F2A2 and F2B. At their present level of sophistication our experiments cannot distinguish between these models or similar ones that can be imagined, nor do we claim that the 11S subunits are necessarily identical in overall protein composition. The availability of a method for producing and isolating these subunits and their multiples under conditions that minimize artifact production should facilitate investigations of the subunit structure of chromatin fibrils.

Note Added in Preparation. While this manuscript was being reviewed, Noll reported that micrococcal nuclease digests rat liver chromatin into a set of nucleoprotein fragments consisting of a fundamental 11.2S unit, a 15.9S dimer, and higher multiples of the fundamental unit (15). Noll concludes, as we do, that chromatin fibrils contain discrete, repeated nucleoprotein subunits. Electrophoretic analyses of DNA isolated from whole digests, calibrated with restriction nuclease fragments of phage ϕX 174 DNA, led Noll to conclude that the 11S units contain DNA that is 200 base-pairs long. This value is considerably larger than our estimate of 120 base pairs for the length of DNA in the 11S units obtained by DNase II disgestion. Differences in the sites of action of the nucleases could possibly explain this discrepancy, although we have found that the gradient patterns of chromatin digested with either DNase II or micrococcal nuclease are identical. Furthermore, Sahasrabuddhe and Van Holde (3) and Clark and Felsenfeld (16) have shown by sedimentation velocity and equilibrium measurements that the major DNA product of extensive chromatin digestion with micrococcal nuclease is 100 to 110 base pairs long.

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