Supranucleosomal Structure of Chromatin: Digestion by Calcium/Magnesium Endonuclease Proceeds via a Discrete Size Class of Particles with Elevated Stability†

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ABSTRACT: When chromatin of rat liver nuclei was examined by in situ digestion by calcium/magnesium endonuclease, the pattern of DNA fragments did not reveal a structural organization above the nucleosomal level. In contrast, the size pattern of soluble chromatin fragments showed a bimodal distribution with a discrete size class of particles exhibiting an elevated stability. Visualization of these stable particles was achieved by gently lysing digested nuclei and sedimentation of the soluble chromatin in density gradients under ionic conditions maintaining the supranucleosomal organization (60 mM NaCl). The sedimentation profiles were bimodal, revealing a stable intermediate fraction and a mono- to oligonucleosomal fraction. The size of the intermediate particles remained constant (~12 nucleosomes) over an extended period of the digestion time, although the DNA continued to be cleaved. When H1 was selectively removed from the soluble chromatin fraction, the particle size distribution exhibited a unimodal profile and the average fragment size continuously decreased upon progressive digestion. This demonstrated the disappearance of stable intermediate particles after H1 depletion. Digestion of erythrocyte nuclei with purified Ca/Mg endonuclease proceeded in a similar way to the in situ digestion of rat liver nuclei with intermediate particles having a size of ~14 nucleosomes. These results, together with the earlier observation of composite particles in a population of H1-containing oligonucleosomes, suggest that intermediate particles were refractory mainly to dissociation but not to digestion and that their preferred size and elevated stability were conferred by histone H1.

The basic fiber of inactive interphase chromatin has a diameter of about 250 Å and shows a periodicity of about 200–250 Å along the fiber (Ris & Chandler, 1963; Yunis & Bahr, 1979). The 250-Å fiber is stable only at "physiological" ionic strength, and, when the salt concentration is lowered, it expands to a less compact nucleofilament (Olins & Olins, 1974; Brasch, 1976). This conformational transition has been investigated in greater detail by hydrodynamic measurements (Renz et al., 1977; Strätling, 1979), neutron scattering studies (Suau et al., 1979), and electron microscopy (Thoma et al., 1979). It is reversible, occurs at 10–20 mM NaCl, and decreases the packing ratio of chromatin by a factor of about 6 (Finch & Klug, 1976). Since the conformational transition was not seen in studies using H1-depleted chromatin fragments (Renz et al., 1977; Strätling, 1979), it was concluded that H1 is an essential structural element for the formation of the 250-Å fiber.

A brief description of recent findings on the nucleosomal architecture is appropriate, as these may have some significance for our understanding of the structure of the 250-Å fiber. Careful analysis of the particles generated by micrococcal nuclease cleavage had indicated that digestion pauses at particles containing H1 and about 165 base pairs (bp) of DNA (Todd & Garrard, 1977; Simpson, 1978). Further digestion results in the formation of particles (core particles) containing about 140 bp of DNA, but lacking H1 (Noll & Kornberg, 1977). From cross-linking studies, the picture has emerged that the globular "head" of H1 contacts the core particle and both "arms" follow the DNA segments linking neighboring nucleosomes (Boulikas et al., 1980). From that is follows that H1 is located at that site of the nucleosome where the DNA enters and leaves the particle. Electron micrographs of fixed chromatin preparations showing that the DNA enters and leaves at the same point confirm this conclusion (Thoma et al., 1979). These electron micrographs had further revealed that at low ionic strength the chain of nucleosomes has the appearance of a zigzag band.

In analogy to the nuclease digestion studies on the nucleosomal architecture, an analytical search was started for evidence for organizational units at the supranucleosomal level. Specifically, nuclear chromatin was subjected to mild digestion followed by sedimentation in density gradients (Renz et al., 1977; Hozier et al., 1977; Strätling et al., 1978). The presence of organizational units of higher order was inferred from the observation of peaks in the sedimentation profile representing chromatin fragments of discrete sizes. Since these studies used size fractionation at the nucleoprotein level, the question whether chromatin particles of discrete sizes occur as these exhibit at barrier to further digestion has not yet received an adequate answer. As an alternative explanation, DNA cleavage may proceed continuously, but digest particles of (a) discrete size(s) exhibit an elevated stability against dissociation. This latter interpretation occurred to us since we showed that "composite" particles, which are assembled from physically distinct mononucleosomes and short-chain oligonucleosomes, represent a significant contribution in the population of digest particles from rat liver nuclei (Strätling, 1979). The size distribution of deproteinized DNA fragments was compared with that of the digest chromatin particles to distinguish between the two alternatives. Here, we report that the DNA appeared to be continuously cleaved, while a defined size class of chromatin particles exhibited an elevated stability, as manifested by a discrete peak in the sedimentation profile of the soluble digest particles. Histone H1 appeared to be the main structural element to provide the elevated stability of these intermediate particles. In this context, a central feature of the present study is the use of Ca/Mg endonuclease from

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rat liver, which lacks the activity of micrococcal nuclease to degrade the cleavage fragments from the ends (Noll & Kornberg, 1977; Ishida et al., 1974). This provided that the DNA binding sites of histone H1 were not degraded and that the stabilizing function of H1 was not impaired in Ca/Mg endonuclease digest particles.

Experimental Procedures

Isolation and Digestion of Rat Liver Nuclei. For preparation of nuclei from rat liver, livers were rapidly excised from male Wistar rats, minced with scissors in buffer A, and homogenized twice for 30 s in this buffer by using the large shaft of an Ultra-Turrax blender (Janke and Kunkel, Freiburg) at 30% of maximal speed. Buffer A contained 0.5 M sucrose, 1.8 mM $MgCl_2$, 5 mM Tris-HCl, 25 mM KCl, 0.14 mM spermidine, 2 mM EGTA, and 0.5 mM phenylmethanesulfonyl fluoride, pH 7.5. The homogenate was filtered through one layer of cheesecloth, followed by another passage through four layers, and then centrifuged for 10 min at 9000 rpm in the HB-4 rotor of a Sorvall centrifuge. The pelleted fraction was homogenized in buffer B (2.2 M sucrose, 1.8 mM $MgCl_2$, 10 mM Tris-HCl, 25 mM KCl, 0.14 mM spermidine, and 0.5 mM EGTA, pH 7.5) by using a Teflon–glass potter. The suspension was then layered onto a 12-mL cushion of buffer B and centrifuged for 35 min at 17 000 rpm in the Beckman SW27 rotor. The pelleted nuclei were washed once in buffer C (1.8 mM $MgCl_2$, 5 mM Tris-HCl, 25 mM KCl, and 0.14 mM spermidine, pH 7.5) and then suspended in buffer "O" of Chevaillier & Philippe (1973) to give an optical density of 100 at 260 nm. Aliquots of the nuclear suspension were then subjected to nucleolysis by endogenous Ca/Mg endonuclease at 37 °C for various lengths of time. The digestion reaction was terminated by adding EDTA and EGTA to 2.7 mM each and NaCl to 35 mM (final monovalent cation concentration 60 mM). After the suspension was chilled on ice, 50 μg/mL pancreatic RNase (made DNase free by incubation for 10 min at 85–90 °C) was added. The material rendered acid soluble by Ca/Mg endonuclease was assayed by measurement of the optical density at 260 nm (multiplied with 0.6 to correct for the hyperchromic effect), following precipitation of aliquots of the nuclear suspension with 0.7 M perchloric acid.

Secondary Digestion by S1 Nuclease. To subject rat liver nuclei digested already by Ca/Mg endonuclease to a secondary digestion by S1 nuclease, nuclei were washed twice with S1 buffer (100 mM NaCl, 1 mM $ZnCl_2$, and 10 mM sodium acetate, pH 5.0) and resuspended in this buffer at the starting concentration of nuclei (Wiegand et al., 1975). Digestion by S1 was carried out at 37 °C for 1 h with 50 000 units of nuclease/mL of nuclei suspension. The reaction was stopped by chilling on ice.

Extraction of Ca/Mg Endonuclease. Extraction of Ca/Mg endonuclease from rat liver was performed according to a modification of the procedure described by Yoshihara et al. (1975). Rat liver nuclei prepared in Mg–EGTA buffer were first washed in 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 50 mM Tris-HCl, pH 7.5, and then extracted with 350 mM NaCl, 1 mM DTT, and 50 mM Tris-HCl, pH 7.5, using the loose pestle of a Dounce homogenizer. The homogenate was centrifuged in the Beckman 60Ti rotor at 50 000 rpm for 2 h. The clear supernatant obtained was dialyzed against 0.5 mM $MgCl_2$, 1 mM DTT, and 20 mM Tris-HCl, pH 7.5, and used in subsequent digestion experiments.

Isolation and Digestion of Erythrocyte Nuclei. Hen erythrocyte nuclei were prepared following the procedure described by Shaw et al. (1976) but reducing the Nonidet P-40 concentration to 0.2%. Digestion of erythrocyte nuclei by rat liver endonuclease was performed at 37 °C in a total volume of 500 μL containing 400 μL of the Ca/Mg endonuclease preparation (extracted from 2.8×10^8 rat liver nuclei), erythrocyte nuclei at a concentration of 11 OD_{260nm}, 1 mM DTT, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl, and 20 mM Tris-HCl, pH 7.5. The reaction was stopped by making the solution of 10 mM in EDTA and 2 mM in EGTA.

Chromatin Fractionation. The nuclear digests were dialyzed overnight against 60 mM NaCl, 5 mM Tris-HCl, pH 7.5, and 1 mM EDTA (STE buffer). The lysed nuclei were separated by centrifugation for 15 min at 12 000 rpm in the Sorvall HB-4 rotor (23 000*g*) into a pelleted fraction and a solubilized chromatin fraction. Aliquots of the latter fraction were layered onto isokinetic sucrose gradients in STE buffer (c_t = 5%, c_r = 26.9%, V_m = 9.4 mL, particle density 1.51 g cm^{-3}; McCarty et al., 1974) and centrifuged in the Beckman SW40 rotor at 40 000 rpm and 4 °C for 1.5 or 2 h. The gradients were fractionated and monitored for absorbance at 254 nm by use of a flow-through cell (ISCO). The term "soluble chromatin" was used to describe the chromatin fraction recovered from the gradient.

For depletion of histone H1 from the solubilized chromatin fraction, a modification of the method described by Thoma et al. (1979) was used. The solubilized fraction recovered after dialysis against 60 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, and 2.5 mM sodium phosphate buffer, pH 7.0, was adjusted to 100 mM NaCl and 10 mM phosphate buffer. Then, the equilibrated exchange resin AG 50W-X2 was added to give a ratio of 4 mL of resin/mL of solubilized chromatin at A_{260} of 100. The suspension was shaken for 2 h at 4 °C and then the resin was removed by low-speed centrifugation. Histone gels from the supernatant fraction showed that this treatment removed 90% of histone H1. The amount of solubilized chromatin recovered after H1 depletion varied between 72 and 84%.

DNA Extraction and Gel Electrophoresis. DNA was extracted from 20-μL aliquots of the nuclear suspensions following the procedure described by Britten et al. (1974). DNA samples in Tris–phosphate buffer (Loening, 1969) and 30% glycerol were electrophoresed in 1% agarose (Bio-Rad) slab gels by using the same buffer system. The gels were stained with ethidium bromide at 1 μg/mL and photographed under ultraviolet illumination.

Results

In Situ Digestion of Rat Liver Nuclei by Ca/Mg Endonuclease. Analysis by DNA Gels. Rat liver nuclei prepared in Mg–EGTA buffer were subjected to digestion in situ by the endogenous Ca/Mg endonuclease for various lengths of time, and the purified DNA was electrophoresed on native agarose gels. The pattern of cleaved fragments shown by the ethidium bromide staining in Figure 1A revealed no evidence of discrete higher order bands. Apparently, cleavage by Ca/Mg endonuclease produced a continuum of fragment sizes. Up to nine bands could be counted, above which individual fragments were not resolved. For examination of the rate of both single-strand and double-strand cleavages, Ca/Mg endonuclease digested nuclei were subjected to a secondary digestion by single-strand specific nuclease S1. Although a comparison between Figure 1, A and B, indicated that early during digestion single-strand cleavages were produced at a much faster rate than double-strand cuts, the gels in Figure 1B did not reveal supranucleosomal bands either. These results indicate that Ca/Mg endonuclease did not recognize a general regular supranucleosomal structure or organizational element(s). However, that does not imply that the chromatin

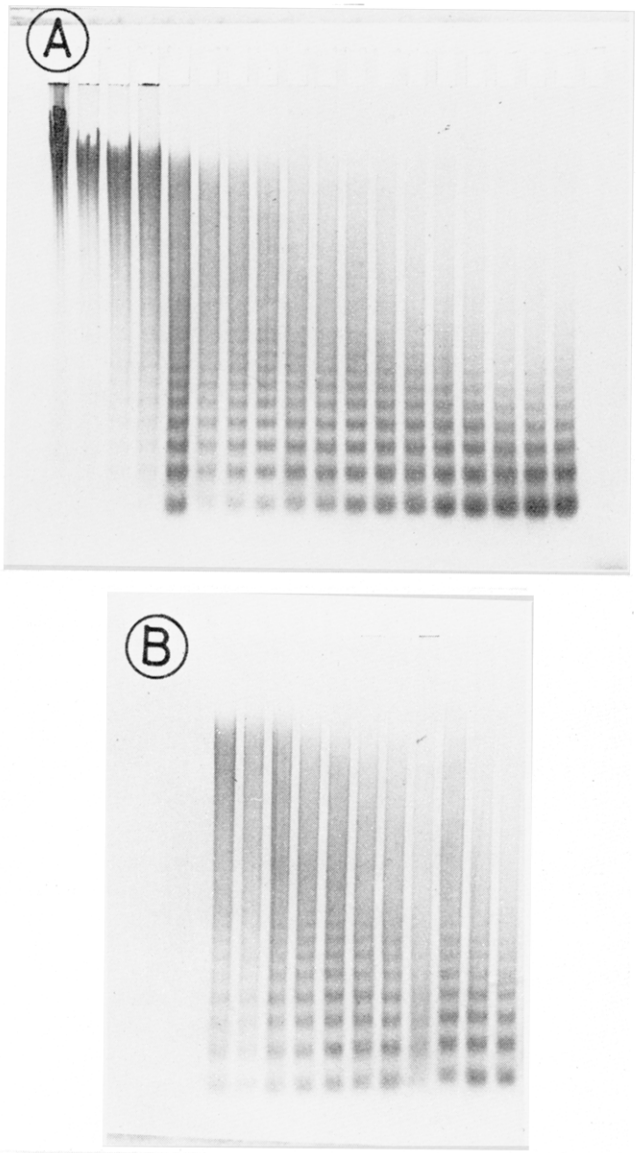

FIGURE 1: Gel electrophoresis of DNA fragments produced by in situ digestion of rat liver nuclei by Ca/Mg endonuclease. (A) Endonuclease digest fragments examined on a 1% agarose gel. The time of digestion, advancing from left to right, was 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, and 44 min. Electrophoresis was from top to bottom. The sample load was 8 μg of DNA per slot. (B) Digest fragments from nuclei subjected to a secondary digestion by S1 nuclease. The time of digestion by Ca/Mg endonuclease, advancing from left to right, was 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 min.

fragments generated by Ca/Mg endonuclease digestion similarly exhibit a continuous size distribution, as the population of digest particles may contain (a) discrete size class(es) of "composite" particles which show an elevated stability against dissociation (Strätling, 1979).

Analysis by Sedimentation of Digested Chromatin Fragments. For investigation of the supranucleosomal structure at the chromatin level, nuclei have to be isolated under the mildest conditions and have to be lysed gently under ionic conditions maintaining the supranucleosomal structure. Thus, rat liver nuclei were isolated in an ionic milieu (1.8 mM Mg^{2+}, 25 mM K^+, and 0.14 mM spermidine) optimally retaining native chromatin structure, as judged from electron micrographs (Chevaillier & Philippe, 1973). In addition, rat liver nuclei prepared in Mg^{2+} buffer retained a 3- to 4-fold higher activity of Ca/Mg endonuclease, as compared to polyamine-stabilized nuclei (Hewish & Burgoyne, 1973) (results not

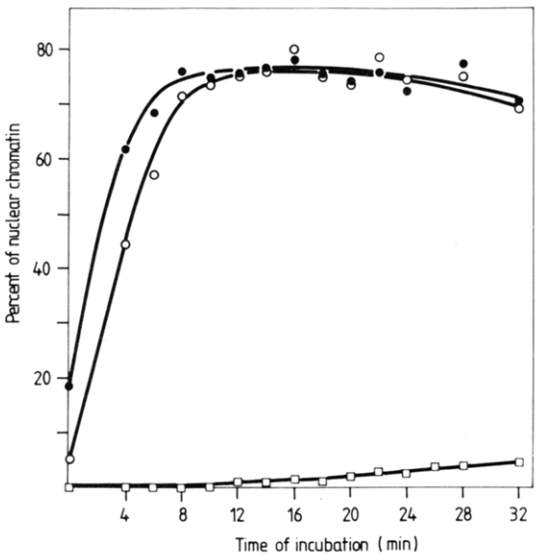

FIGURE 2: Solubility of rat liver chromatin digested in situ by Ca/Mg endonuclease. (●) Solubilized chromatin fraction obtained after low-speed centrifugation of the lysed nuclear preparation. (○) Soluble chromatin fraction recovered from the gradient after sedimentation in isokinetic density gradients. (□) DNA rendered acid soluble.

shown). Following digestion, nuclei were lysed by dialysis against a buffer containing 60 mM NaCl. This NaCl concentration is well above that (10–20 mM NaCl) at which the chromatin fiber undergoes the conformational transition to the more expanded nucleofilament (Strätling, 1979; Thoma et al., 1979). Low-speed centrifugation of the lysed nuclear suspension yielded a solubilized chromatin fraction which retained only marginal turbidity (ratio of A_{320nm} vs. A_{260nm} = 0.01). The solubilized fraction was then sedimented in isokinetic sucrose gradients, and the recovered material was designated as "soluble" in 60 mM NaCl buffer. Without incubation, 19% and 5% of the nuclear chromatin appeared in the solubilized and soluble fraction, respectively, indicating that some endonuclease cleavage had occurred already during nuclei preparation (Figure 2). The difference in the amount of nuclear chromatin yielded in the low-speed supernatant fraction and that recovered from the density gradient accounted for larger fragments sedimenting to the bottom of the centrifuge tube. When nuclei were incubated at 37 °C, the amount of nuclear chromatin recovered in the solubilized and the soluble fraction rose rapidly to reach a plateau value of about 75% within 6 min and 10 min of incubation, respectively. This demonstrates the remarkable fact that about three-fourths of endonuclease-digested chromatin was soluble in 60 mM NaCl buffer. This result cannot be attributed to the use of the Ca/Mg endonuclease from rat liver, as similar results were obtained after digestion by micrococcal nuclease (Strätling et al., 1978). When nuclei digested for 14 min were pelleted and resuspended in 60 mM NaCl buffer, only about 4% of nuclear chromatin were recovered in the solubilized and the soluble fraction. This suggests that the high yield of soluble chromatin has to be attributed to the mild procedure (dialysis) to lyse the nuclei. Acid-soluble material was not produced within the very first 10 min of digestion and was generated, thereafter, at a very low rate (Figure 2). Since the endonuclease was reported to lack an exonucleolytic activity (Ishida et al., 1974), this low rate is likely to reflect the activity of exonucleases present in rat liver nuclei.

The size distribution of the soluble chromatin particles as analyzed by sedimentation in isokinetic sucrose gradients is shown in Figure 3A. The sedimentation profile at 0 min of incubation revealed large chromatin particles increasing con-

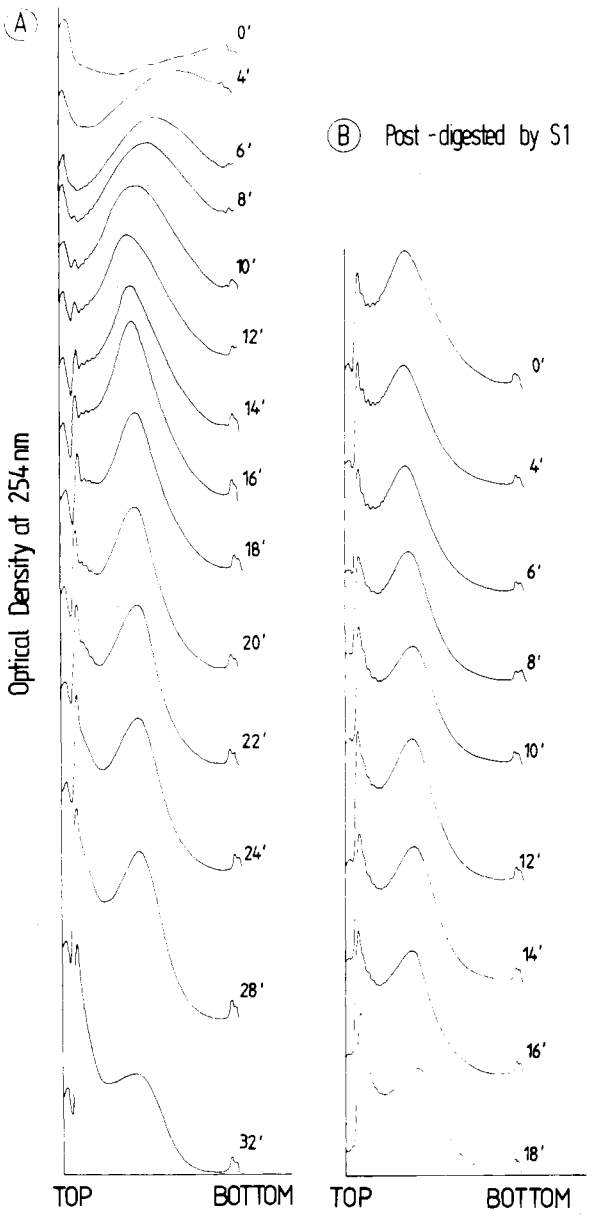

FIGURE 3: Sedimentation of soluble chromatin particles produced by in situ digestion of Mg–EGTA-stabilized rat liver nuclei by Ca/Mg endonuclease. (A) Soluble endonuclease digest particles analyzed on isokinetic sucrose gradients. Sedimentation (for 2 h) is from left to right. Gradients were loaded each with 200 μL of the solubilized chromatin fraction obtained by low-speed centrifugation. (B) Soluble endonuclease digest particles from nuclei postdigested by S1 nuclease. The time of digestion (min) by Ca/Mg endonuclease is indicated in the figure.

tinously in their apparent molecular weight. When nuclei were incubated at 37 °C, the average size of the soluble chromatin fragments rapidly decreased within the first 12 min, but the profiles essentially remained unimodal. At 12 min of incubation, the particle size distribution started to exhibit a bimodal profile, showing a fraction of intermediate particles, as manifested by a discrete peak, and a mono- to oligonucleosomal fraction. When nuclei were incubated further, the intermediate particles were gradually processed to mono- and oligonucleosomes, as revealed by a gradual decrease in the fractional amount of chromatin in the intermediate peak. Most remarkably, the position of this peak in the profile remained constant up to 32 min of incubation, indicating an invariant average size of the intermediate particles. The manifestation of digestion intermediates as a discrete peak in the gradient profiles was not expected from the DNA gels. Therefore, it

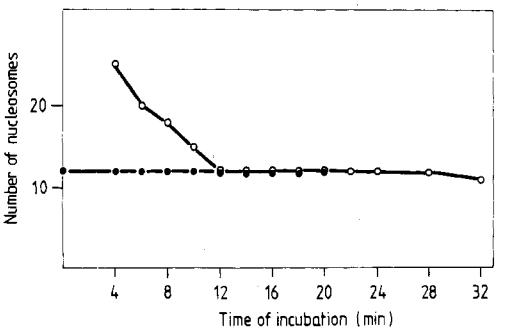

FIGURE 4: Size of the intermediate particles. The particle size on top of the intermediate peak and of the peak representing larger soluble fragments early during digestion was expressed in terms of number of nucleosomes. (○) Ca/Mg endonuclease digest; (●) Ca/Mg endonuclease digest further postdigested by S1 nuclease.

was appropriate to conclude that the intermediate particles did not represent a barrier to digestion but exhibit an elevated stability against dissociation, which is conferred by protein–DNA and/or protein–protein interactions.

To exclude the possibility that ribonucleoprotein particles contributed to the sedimentation profiles monitored, we routinely subjected the suspension of endonuclease digested nuclei to a secondary digestion of pancreatic RNase. A chemical analysis showed that following this treatment RNA was not detectable in the intermediate peak fraction (RNA/DNA ratio below 0.01:1). The possibility just mentioned is further excluded by the observation that the optical density material under the intermediate peak was processed quantitatively to the mono- and oligonucleosomes after prolonged endonuclease digestion.

The size distribution of soluble chromatin particles from nuclei subjected to a secondary digestion by S1 nuclease differed significantly from the respective Ca/Mg endonuclease digested ones (Figure 3B). The profile at zero time of incubation exhibited already a bimodal profile, indicating that during nuclei purification Ca/Mg endonuclease had nicked chromatin DNA extensively. When the time of digestion by Ca/Mg endonuclease was increased, the profile of S1-postdigested nuclei revealed that with progressive digestion intermediate particles were processed at a very low rate to mono- and oligonucleosomes. Thereby, the profiles from endonuclease digested nuclei and the respective profiles from nuclei subjected to a secondary S1 digestion approached each other gradually. From this follows that the single-strand cleavages generated by the Ca/Mg endonuclease during nuclei purification were preferentially extended to double-strand cuts during the first period of incubation.

The profiles of the isokinetic sucrose gradients shown in Figure 3 resolved oligonucleosomal subpeaks up to the octamer. The distances between neighboring subpeaks proved to be constant (with the exception of an increased distance between monomer and dimer). Thus, an extrapolation to that region of the profile not resolving oligonucleosomal subpeaks allowed us to trace the size of Ca/Mg endonuclease cleaved particles as a function of the time of digestion (Figure 4). At 12 min of incubation, the particles recorded on top of the peak in the sedimentation profile reached a size of about 12 nucleosomes and continued to exhibit this size up to 32 min. When the nuclei was subjected to a secondary digestion by S1 nuclease, the top of the intermediate peak corresponded to particles having a size of about 12 nucleosomes from the onset of incubation. Sedimentation for longer periods of time confirmed the average size of 12 nucleosomes in intermediate particles. A careful inspection of the profiles in Figure 3 revealed that the sedimentation coefficient of each oligonucleosomal subpeak

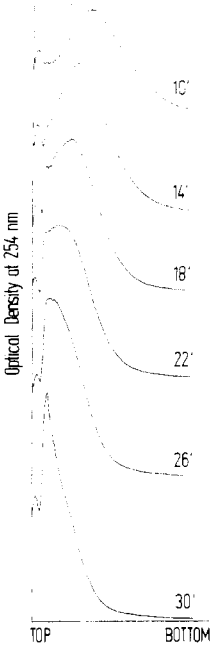

FIGURE 5: Effect of H1 depletion on the size distribution of soluble chromatin fragments. Nuclei digested in situ by Ca/Mg endonuclease for the times indicated were lysed by dialysis. The solubilized fraction obtained was selectively depleted of histone H1 and then sedimented in isokinetic gradients.

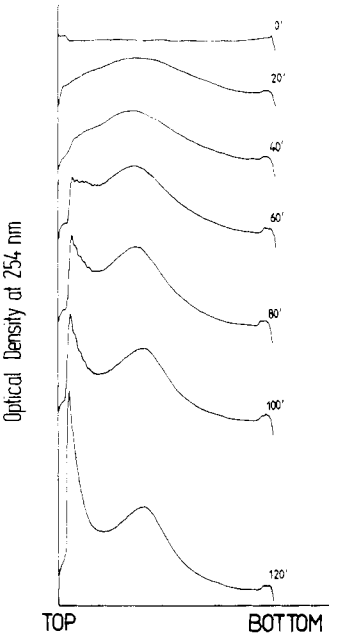

FIGURE 6: Digestion of hen erythrocyte nuclei by Ca/Mg endonuclease from rat liver. Erythrocyte nuclei were digested with a purified preparation of Ca/Mg endonuclease from rat liver for the times indicated. The size pattern of the cleaved soluble particles was analyzed by density gradient sedimentation (for 1.5 h).

as well as of the intermediate peak increased slightly with time of digestion. From this coordinate increase it follows that the elevated sedimentation coefficient of the intermediate peak cannot be attributed to an increase in the number of nucleosomes (see also Figure 4). This, in turn, invalidates the possibility that the intermediate peak at later stages of digestion resulted from an aggregation of particles occurring after digestion. Instead, we favor the explanation that some nonhistone proteins have relatively weak affinities to mononucleosomes and may redistribute to larger particles with time of digestion, thus increasing their sedimentation coefficients.

Effect of H1 Removal and Dilution. Since the DNA gels in Figure 1 revealed no evidence of supranucleosomal bands, we anticipated that the elevated stability of the intermediate particles was conferred by DNA–protein and/or protein–protein interactions. For investigation of the involvement of histone H1 in this stabilizing function, histone H1 was selectively removed from the solubilized chromatin fraction before sedimentation in isokinetic sucrose gradients. Figure 5 shows that the distribution of H1-depleted fragments exhibited a unimodal profile at any time of digestion. The average fragment size decreased continuously with progressive digestion. A comparison with the profiles in Figure 3 clearly reveals the effect of H1 removal on the size distribution of the chromatin digest fragments. This suggested that H1 is an essential element to stabilize the intermediate particles. This result further confirms earlier studies implicating H1 as a cross-linking element in "composite" oligonucleosomal particles (Strätling, 1979).

Since the integrity of the intermediate particles was primarily conferred by noncovalent interactions, most probably those of H1, it was of interest to study the sensitivity of the stability of the intermediate particles to dilution. The bimodal profiles shown in Figure 3 were obtained when rat liver nuclei were digested and lysed at an A_{260nm} of 100. When the digested nuclei were diluted up to an A_{260nm} of 5, the sedimentation profiles retained a bimodal profile, but small changes in detailed features of the profiles occurred. The average size of the intermediate particles decreased to about 10 nucleosomes, and, simultaneously, chromatin material of the intermediate peak shifted to the mono- and oligonucleosomal fraction (data not shown). This shows that the elevated stability of intermediate particles from rat liver was slightly sensitive to dilution. Since the intermediate particles were, in part, "composite" particles (Strätling, 1979), their size may be regarded to be subject to an association–dissociation equilibrium which will be shifted to dissociation upon dilution. Thus, the reduction in the average size of the intermediate particles and the shift of chromatin from the intermediate fraction to the mono- and oligonucleosomal fraction may be explained by a disassembly into mononucleosomes and shorter chain oligonucleosomes under more dilute conditions.

Digestion of Erythrocyte Nuclei by Rat Liver Ca/Mg Endonuclease. To exclude the possibility that intermediate particles of a discrete size were preferentially generated, since the Ca/Mg endonuclease is located nonrandomly along the chromatin fiber, we digested nuclei from hen erythrocytes for various lengths of time with an extract of rat liver nuclei containing the Ca/Mg endonuclease activity. The size distribution of cleaved soluble particles exhibited a bimodal profile from 40 min up to at least 140 min of incubation time (Figure 6). This result invalidated the possibility that intermediate particles of a preferred size were cleaved from chromatin due to a nonrandom distribution of the enzyme (Marshall, 1977). Instead, it denoted the appropriateness of our earlier interpretation that the preferred occurrence of discretely sized intermediate particles was caused by their elevated stability. By determining the particle size on top of the intermediate peak, we obtained an estimate of, on the average, about 14 nucleosomes per particle. This indicated that intermediate particles from hen erythrocytes were slightly larger than those of rat liver. In this respect, it is appropriate to note that the digestion of erythrocyte nuclei was performed at one-tenth the concentration of nuclei (A_{260nm} 10) compared to that of rat liver nuclei. Since the sedimentation profiles obtained from erythrocyte nuclei exhibited very clearly a bimodal appearance at this reduced chromatin concentration, the intermediate particles from erythrocyte nuclei seem to be more stable than

those from rat liver. This suggestion would be in accord with the tighter mode of packaging of chromatin in this cell type. Nevertheless, despite the higher degree of compaction, 90–100% of the chromatin in erythrocyte nuclei were rendered soluble by the dialysis procedure to lyse the nuclei.

Discussion

The results obtained demonstrate the presence of a discrete size class of intermediate chromatin particles with elevated stability appearing during in situ digestion of rat liver nuclei by Ca/Mg endonuclease and during digestion of erythrocyte nuclei by Ca/Mg endonuclease from rat liver. This conclusion is based on analysis of the size pattern of chromatin fragments by sedimentation in isokinetic density gradients. During a time course of digestion with each type of nuclei, the sedimentation profile was bimodal with a nearly invariant size of the intermediate particles. The particles from rat liver nuclei contained, on the average, about 12 nucleosomes, while those from hen erythrocytes were slightly larger (~14 nucleosomes). In a recent study of the mode of digestion of lymphocyte nuclei by micrococcal nuclease, a heterogeneous population of intermediate particles of similar size (on the average, ~12 nucleosomes) has been described (Renz, 1979). Earlier measurements (Strätling et al., 1978) of the size of the intermediate particles (8 nucleosomes) have been obfuscated by contaminating ribonucleoprotein particles—a problem rigorously ruled out in the present study.

Since the DNA gels did not reveal higher order bands, the DNA appeared to be continuously cleaved. From this we conclude that the intermediate particles exhibited an elevated stability even though their DNA was fragmented to smaller sizes. This view is supported by the DNA gels of selected fractions of higher order particles (Strätling et al., 1978). These gels show a sharp high molecular weight front but a long tail of fragments of smaller sizes. The above conclusion is further reached considering the disaggregation of chromatin digest fragments into much smaller sizes produced by the removal of histone H1. This latter finding suggests that the elevated stability of the intermediate particles was conferred by histone H1. In this respect, the intermedate particles featured the properties of particles (composite particles) described earlier to occur in nuclear digests of rat liver (Strätling, 1979). Composite particles dissociate into smaller nucleosomal chains at either very low or very high ionic strength. From depletion and reconstitution studies, it was concluded that the stability of these particles was mediated by H1.

We turn our attention now to the mechanism of how discretely sized chromatin fragments were generated and in what respect they represent features of native chromatin structure. From the continuous size distribution of the deproteinized DNA fragments (see Figure 1; Wu et al., 1979) and of the H1-depleted digest fragments, we conclude that the digestion intermediates are mainly refractory to dissociation, but not to digestion. We have thus answered the question raised in the introduction. The notion that the endonuclease cleaves preferentially at nucleosomes on adjacent turns (Thoma et al., 1979) or at periodically arranged, "dislocated" nucleosomes (Suau et al., 1979) is not supported by our observations and cannot be invoked as an explanation for the occurrence of stable intermediate particles. Further, the DNA gels exclude that after a certain stage of digestion a conformational change of the digest fragments prevented further DNA cleavage. Finally, the sedimentation profiles and the constant size of the intermediate fragments exclude that these particles result from aggregation of particles occurring after a certain stage of digestion. For these reasons, we wish to conclude that the preferentially stabilized intermediate particles represent a feature of native chromatin structure.

This in turn leads us to the discussion of the structure of the 250-Å fiber. Our studies showed that the fiber exhibits a random susceptibility to DNA cleavage by Ca/Mg endonuclease. This seems to rule out models which assume a periodic array of larger beads with an elevated susceptibility to nuclease digestion between these beads. Instead, it is more likely that the stability of the 250-Å fiber is periodically organized. Upon random DNA cleavage of the fiber, it disassembles into fragments which reflect the periodically arranged stability of the fiber; i.e., fragment sizes are preferred which represent a unit size in the stability organization. This interpretation receives support from a recent electron microscopic study on the disassembly of the 250-Å fiber upon gradual lowering of the mono- and divalent cation concentration or by tRNA treatment (Pruitt & Grainger, 1980). Discretely sized units appear as intermediates during reduction of ionic strength. Similarly, the discontinuous appearance of the 250-Å fiber observed by other investigators (Kiryanov et al., 1976; Renz et al., 1977; Rattner & Hamkalo, 1978; Zentgraf et al., 1980) may be explained by a partial disassembly of the fiber disclosing the sites ("weak" sites) between the more stabilized units. We are unable to explain why other electron microscopic studies (Finch & Klug, 1976; Thoma et al., 1979) did not reveal a discontinuous organization of the 250-Å fiber, but an answer may be sought considering details of the nuclei preparation (e.g., use of polyamine stabilized nuclei; Billet & Hall, 1979) or of the electron microscopic methodology (Labhart & Koller, 1980).

In summary, it may be inferred from our observations that the 250-Å fiber features a periodic arrangement of the fiber stability. The biochemical and structural basis of this periodic arrangement, however, remains to be elucidated. The only conclusion which may be drawn from the present study is that this periodicity is likely to be expressed at the level of H1–H1 and/or H1–DNA interactions. This suggestion receives support from recent studies on the self-polymerization of H1 after exposure to cross-linking reagents (Itkes et al., 1980). An analysis of the size distribution of the cross-linked aggregates revealed that polymers containing 12, 24, etc., H1 molecules preferentially occur. Further, the disassembly into higher order units by partial H1 removal (Pruitt & Grainger, 1980) opens the possibility that H1 was first removed at the less stabilized ("weak") sites of the fiber. Although further studies are needed to expand our knowledge on the function of H1, the observations mentioned above may give a justification for the hypothesis which claims that H1 features a periodicity in its interactions along the length of the 250-Å fiber. This concept is different from the models proposed by Finch & Klug (1976) and by Renz et al. (1977), but it bears some features inherent to both of those.

Acknowledgments

We thank M. Planken for helpful technical assistance. We are also grateful to one of the reviewers, who suggested the performance of the H1-depletion experiment in the way of removing H1 from the digest particles rather than digesting H1-depleted nuclei.

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Translational Mobility of Glycophorin in Bilayer Membranes of Dimyristoylphosphatidylcholine†

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ABSTRACT: The translational diffusion of the integral membrane sialoglycoprotein from erythrocyte membranes, glycophorin, incorporated into bilayer membranes of dimyristoylphosphatidylcholine at a protein/lipid molar ratio of 1:4500 was examined by using the fluorescence redistribution after photobleaching technique. A plot of the diffusion coefficient vs. temperature shows a sharp decrease in the rate of diffusion at about 15 °C. This sharp diffusion transition is at a temperature some 9 °C lower than the calorimetrically measured lipid gel–liquid crystalline phase transition temperature of the system. The difference between the diffusion transition temperature and the lipid phase transition temperature is attributed to a localized fluidizing effect of the protein upon the gel phase lipid. The value of the diffusion coefficient above 15 °C was found to be (1–2) × 10^{-8} cm^2 s^{-1}, and below 15 °C it was lower than about 5 × 10^{-11} cm^2 s^{-1}. The fluorescence recovery in the bleached area as a consequence of diffusional redistribution appeared to be due to a single diffusing species at temperatures above 15 °C and due to more than one diffusing species below this temperature.

The translational and rotational diffusion of membrane components is of considerable biochemical and biophysical interest [for a recent review, see Cherry, (1979)]. One approach to the understanding of diffusion in biological membranes is the study of diffusion in "reconstituted" model membranes. In such systems, with a limited number of well-defined components, the interactions of the components with each other and the behavior of the system as a whole are generally fairly well understood. The study of diffusion of membrane-bound proteins in simple model systems could yield important insights into the problem of diffusion of membrane-bound proteins in the far more complex biological membranes. Further, an experimental evaluation of existing theoretical treatments (Saffman & Delbrück, 1975; Saffman, 1976) of diffusion in quasi-two-dimensional systems such as membranes may be useful.

The experimental study of the translational diffusion of proteins in reconstituted membranes is fairly new (Derzko & Jacobson, 1978; Vaz et al, 1979a,b; Smith et al., 1979a; Schindler et al., 1980). These studies have been made possible

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0006-2960/81/0420-1392$01.25/0 © 1981 American Chemical Society