FRACTIONATION OF NUCLEOSOMES BY SALT ELUTION FROM MICROCOCCAL NUCLEASE-DIGESTED NUCLEI

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ABSTRACT

The solubilization of nucleosomes and histone H1 with increasing concentrations of NaCl has been investigated in rat liver nuclei that had been digested with micrococcal nuclease under conditions that did not substantially alter morphological properties with respect to differences in the extent of chromatin condensation. The pattern of nucleosome and H1 solubilization was gradual and noncoordinate and at least three different types of nucleosome packing interactions could be distinguished from the pattern. A class of nucleosomes containing 13–17% of the DNA and comprising the chromatin structures most available for micrococcal nuclease attack was eluted by 0.2 M NaCl. This fraction was solubilized with an acid-soluble protein of apparent molecular weight of 20,000 daltons and no histone H1. It differed from the nucleosomes released at higher NaCl concentrations in content of nonhistone chromosomal proteins. 40–60% of the nucleosomes were released by 0.3 M NaCl with 30% of the total nuclear histone H1 bound. The remaining nucleosomes and H1 were solublized by 0.4 M or 0.6 M NaCl. H1 was not nucleosome bound at these ionic strengths, and these fractions contained, respectively, 1.5 and 1.8 times more H1 per nucleosome than the population released by 0.3 M NaCl. These fractions contained the DNA least available for micrococcal nuclease attack. The strikingly different macromolecular composition, availability for nuclease digestion, and strength of the packing interactions of the nucleosomes released by 0.2 M NaCl suggest that this population is involved in a special function.

KEY WORDS nucleosome fractionation · nucleosome packing · histone H1 interaction with nucleosomes · histone H1 stoichiometry · chromatin condensation mechanism

Chromatin in the interphase nucleus in eukaryotic organisms is organized morphologically in more condensed and less condensed structures. RNA synthesis apparently occurs in the less condensed regions (30), and satellite DNA is thought to be present in condensed chromatin. Recent evidence that more than 85% of the nuclear DNA, including the actively transcribing genes as well as satellite DNA sequences, is complexed with histones in particulate structures called nucleosomes (3, 6, 23, 24, 39, 42, 46, 52) suggests that the more- and less-condensed morphological features of the nucleus may reflect differences in the macromolecular composition and/or the packing of the nucleosomes. The differential condensation is preserved in nuclei isolated in the presence of divalent cations (51), and dilution or chelation of divalent cations causes the native nuclear organi-

zation to be lost (2, 11, 16, 29, 35, 43, 45, 49). Thus, investigation of these possibilities required a procedure for the preparation of micrococcal nuclease-digested nuclei that did not alter the differences in extent of condensation. Such a procedure was developed in the course of investigating the divalent cation-dependent aggregation properties of purified monomeric nucleosomes (50). The perturbation of micrococcal nuclease-digested nuclei prepared under these conditions with increasing concentrations of NaCl showed that at least three basic types of nucleosome packing interactions can be distinguished in rat liver nuclei.

MATERIALS AND METHODS

Purification of Rat Liver Nuclei

Nuclei were isolated by the method of Chevaillier and Philippe (11) from fresh rat livers weighing 7–9 g. After scissor-mincing, the liver was homogenized with two strokes of a motor-driven Potter-Elvehjem homogenizer in 100 ml of 1 mM Tris, 25 mM KCl, 0.9 mM $MgCl_2$, 0.9 mM $CaCl_2$, 0.14 mM spermidine, pH 7.6 (C buffer) containing 2.2 M sucrose. The homogenate was filtered through four layers of cheesecloth and distributed equally in two 70-ml centrifuge tubes which were then filled to the top with C buffer plus 2.2 M sucrose. The nuclei were pelleted by centrifugation in a Spinco 45 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 26,000 rpm at 4°C for 1 h. The supernatant fraction was poured off and the inside of the tube was wiped with a tissue. Nuclear pellets from one liver were resuspended with a homogenizer in 5 ml of C buffer.

Digestion of Purified Nuclei

The nuclei from one rat liver (DNA concentration 1.6–1.8 mg/ml) were treated with 200 units/ml of micrococcal nuclease (Worthington Biochemical Corp., Freehold, N. J.) in a total volume of 5 ml of C buffer containing 1.8 mM $MgCl_2$ (instead of 0.9 mM) at 37°C for 2–20 min. These conditions released 2–18% of the DNA as acid-soluble nucleotides. The digestion was stopped by the addition of 0.5 ml of 10 mM ethylene glycol-bis(β-aminoethylether)-N,N′-tetraacetic acid (EGTA).

Quantitation of Nucleosome and Histone H1 Release

Nucleosome release by increasing concentrations of NaCl was measured by dividing an EGTA-arrested digest into four equal parts. Each part was centrifuged at 3,000 rpm in a Sorvall HB 4 rotor (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) at 4°C for 10 min, and the supernate (S_0) was removed. Each pellet was resuspended in C buffer plus 0.9 mM $MgCl_2$, 1 mM EGTA and either 0.2, 0.3, 0.4, or 0.6 M NaCl and allowed to stand for 20 min on ice. Samples were removed from the suspension for determination of total DNA, and the suspensions were centrifuged again under the same conditions. DNA present in the supernates S_0, S_2, S_3, S_4, and S_6 and in the total suspensions was measured by the diphenylamine procedure (9).

Histone H1 release was quantitated by precipitating aliquots of total digest and each of the supernates S_0, S_2, S_3, S_4, and S_6 with two volumes of 95% ethanol. The precipitated samples were left at −20°C overnight, centrifuged, and taken up in 80 μl of loading buffer for discontinuous sodium dodecyl sulfate (SDS) acrylamide slab gel electrophoresis (25). 5, 10, 20, and 40 μl of each sample was applied in a total volume of 40 μl to a slab gel and electrophoresed as described below. After staining with Coomassie blue and destaining, the gels were photographed and the area under each H1 peak was determined with a Joyce-Loebl densitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England) equipped with an integrator. The linearity of the response of the staining and photographic processes was determined for each slab gel from the dilutions of each sample described above.

Stepwise Elution of Nucleosomes

For determination of the macromolecular composition of the nucleosomes eluted at each NaCl concentration, an EGTA-arrested micrococcal nuclease digest was centrifuged at 3,000 rpm in a Sorvall HB 4 rotor. The supernate (S_0) was removed and the pellet was resuspended in 5 ml of C buffer plus 0.9 mM $MgCl_2$, 1 mM EGTA and 0.2 M NaCl. After 20 min on ice, the suspension was centrifuged as above, the supernate (SS_2) was removed, and the pellet was resuspended in C buffer plus 0.9 mM $MgCl_2$, 1 mM EGTA and 0.3 M NaCl. The process was repeated at each salt concentration.

Sucrose Gradient Experiments

The sedimentation properties of the salt-eluted nucleosomes were analyzed on 11-ml linear 10–30% sucrose gradients formed in C buffer plus the indicated amount of NaCl. 0.2–0.5 ml of the sample was layered onto the gradients, and the latter were centrifuged in a Spinco SW 41 rotor at 35,000 rpm for 18 h at 4°C. 0.8-ml fractions were collected after puncturing the bottom of the tube, and the absorbance at 260 nm of each fraction was measured.

Gel Electrophoresis

For size analysis of the DNA present in the fractionated nucleosomes, the DNA was purified by the method of Noll (38) and 5 or 10 μg of each of the samples was electrophoresed on 6% acrylamide slab gels (32) at 50

mA for 6 h and compared with DNA purified from the total digest. The gels were stained with 2 μg/ml ethidium bromide in water for 30 min and photographed on a UV light box (Ultra-Violet Products Inc., San Gabriel, Calif.) with a Kodak 23A filter.

Total proteins of various fractions of nucleosomes or digests were analyzed on 12.5% discontinuous SDS-acrylamide slab gels (25) as described previously (50).

Histones and other acid-soluble proteins were extracted as described previously (50). They were analyzed by two-dimensional electrophoresis with triton-urea-acetic acid-acrylamide (1, 14, 18) in the first dimension and a discontinuous SDS acrylamide electrophoresis in the second dimension. The samples were electrophoresed in slab gels in triton-acetic acid-urea as described previously (50). The individual tracks were cut out with a knife and were equilibrated over the period of 1 h with three changes of sample-loading buffer for discontinuous SDS acrylamide slab gels (25). The tracks were then frozen until they were electrophoresed in the second dimension. For the second dimension, each track was laid across the top of a slab gel consisting of a 12.5% running gel and a 2-cm stacking gel, both prepared as described previously (25). The track was sealed in place with 1% agarose in the sample-loading buffer (25).

RESULTS

Nuclear Morphology and Nucleosome Release Related to Ionic Conditions

Nuclei that have been isolated at neutral pH in buffers containing divalent cations retain the morphology characteristic of the nucleus in situ. Fig. 1 a shows a phase micrograph of rat liver nuclei isolated by a procedure that was optimized for the purpose of preserving native morphology (11). The nucleoli and condensed chromatin are prominent as dense inclusions. Fig. 1 b shows the effect of 5 mM EDTA on the morphology of nuclei from the same preparation. The morphological difference in extent of chromatin condensation is lost in procedures that utilize EDTA to chelate divalent cations and solubilize chromatin or nucleosomes. The nucleoli are still prominent but differences in the extent of chromatin condensation have disappeared.

This loss of differential chromatin condensation is not observed when nuclei are digested with micrococcal nuclease under conditions where divalent cations are not removed from the medium. Fig. 2 shows phase micrographs of rat liver nuclei before and after digestion with micrococcal nuclease to the mononucleosome stage (18% acid solubilization of the DNA) where the digestion was carried out in the presence of both Ca^{2+} and

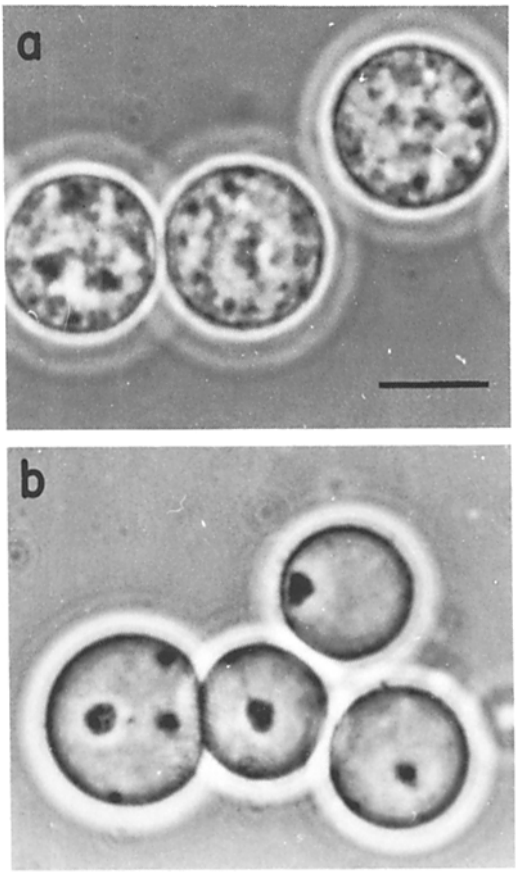

FIGURE 1 Rat liver nuclear morphology in the presence and absence of divalent cations. Nuclei isolated by the method of Chevaillier and Philippe (11) were observed in C buffer (*a*) and in C buffer plus 5 mM EDTA (*b*). Phase micrograph. Bar, 5 μm.

Mg^{2+} and stopped by selective chelation of Ca^{2+} with EGTA (50). The digested nuclei (Fig. 2 b) show subtle changes in nucleolar morphology, but gross nuclear morphology with respect to differences in the extent of chromatin condensation is changed very little. This observation suggested that the structures and interactions responsible for the characteristic features of nuclear morphology had not been disrupted by the digestion procedure although the DNA strand had been broken. One test for this possibility was to determine whether 11 S nucleosomes were released from nuclei digested by this procedure. For this purpose, a micrococcal nuclease digest of nuclei was arrested with EGTA and was divided into three parts. One part was not further treated; EDTA to a final concentration of 5 mM was added to the second

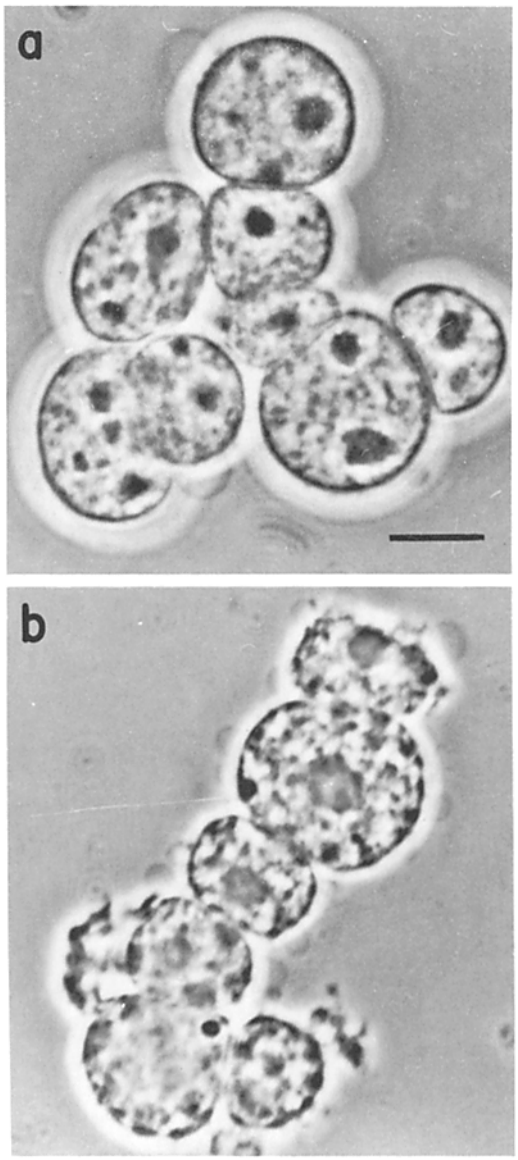

FIGURE 2 Rat liver nuclear morphology before and after EGTA-arrested micrococcal nuclease digestion. Nuclei isolated as in Fig. 1 were observed in C buffer before digestion (*a*) and after EGTA-arrested micrococcal nuclease digestion to the mononucleosome stage (18% acid solubilization of the DNA) in C buffer plus 1 mM $MgCl_2$ and 1 mM EGTA (*b*). Phase micrograph. Bar, 5 μm.

part; the third part was dialyzed against C buffer containing 0.6 M NaCl, a procedure known to release all the nucleosomes and histone H1 (50). The amount of 11 S nucleosomes released by these procedures was compared on sucrose gradients as shown in Fig. 3. No detectable 11 S nucleosomes were released from the EGTA-treated sample, and 15% of the DNA was released in the 11 S peak by EDTA. Impermeability of the nuclear membrane to release of nucleosomes in the EGTA case was probably not the

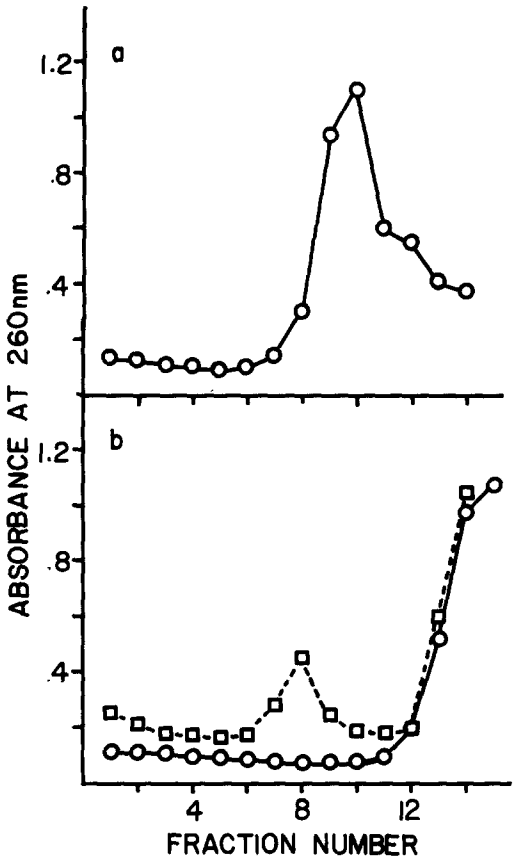

FIGURE 3 Release of nucleosomes from micrococcal nuclease-digested nuclei as a function of ionic conditions. An EGTA-arrested digest of rat liver was divided into three parts. One part was not further treated; EDTA to a final concentration of 5 mM was added to the second part, and the third part was dialyzed overnight against C buffer plus 0.6 M NaCl. Aliquots from the three samples containing the same amount of DNA were centrifuged through 11-ml 10–30% sucrose gradients prepared, respectively, in C buffer plus 1 mM $MgCl_2$ and 1 mM EGTA, C buffer plus 5 mM EDTA and C buffer plus 0.6 M NaCl in a SW 41 rotor at 35,000 rpm for 18 h at 4°C. Sedimentation was from right to left. The absorbance at 260 nm for each 0.8-ml fraction collected from the bottom was determined. (*a*) 0.6 M NaCl-treated digest. Soluble nucleotides are not present at the top of this gradient because of the dialysis step. (*b*) (○——○) EGTA-arrrested digest; (□- -□) EDTA-treated digest.

reason for this result since removal of nuclear membranes by Triton X-100 had no effect.

These observations show that forces in addition to the continuity of the DNA strand are important in the packing of nucleosomes inside the nucleus. Presumably, the packing involves interactions between nucleosomes, or between a nucleosome and internucleosome DNA, or between nucleosomes and nuclear matrix structures. Studies with purified chromatin have indicated that cross-linking of chromatin strands or packing of the chromatin fiber may be mediated by histone H1 (7, 8, 12, 17, 31, 36, 44, 48). The absence of both the release of 11 S nucleosomes and extensive change in nuclear morphology after EGTA-arrested micrococcal nuclease digestion of nuclei suggests that this digestion procedure has not affected the packing forces by these criteria. The nature of the packing interactions can thus be investigated by quantitation of the release of nucleosomes from digested nuclei under a variety of perturbing conditions.

Elution of Nucleosomes and H1 with NaCl

Differences in the extent of chromatin condensation in the nucleus are known to gradually disappear with increasing NaCl concentrations, and nuclear morphology collapses in 0.6 M NaCl (29, 43) coincident with complete dissociation of histone H1 (41). Previous to H1 dissociation, the process is reversible but only when divalent cations are present (29). The correlation of nucleosome and H1 release from digested nuclei as a function of increasing NaCl concentration should thus show what role H1 plays in mediating internucleosome interactions. Nucleosome and H1 release from EGTA-arrested micrococcal nuclease digests of nuclei were quantitated in experiments designed as diagrammed in Fig. 4. Digested nuclei were treated with C buffer containing either 0.2, 0.3, 0.4, or 0.6 M NaCl. The DNA solubilized by each NaCl concentration was measured and expressed as a percentage of the DNA present in the total digest. H1 solubilized was determined by quantitative SDS acrylamide gel electrophoresis. Fig. 5 shows the pattern of release of nucleosomes and of H1. DNA was gradually eluted by increasing NaCl concentrations with less than 20% solubilized in 0.2 M NaCl and close to 100% solubilized at 0.4 and 0.6 M NaCl. The pattern of DNA release with increasing NaCl concentrations did not vary appreciably with the extent of digestion in the range between 2 and 18% solubilization of the DNA. This indicates that the DNA available for micrococcal nuclease digestion does not play a major role in stabilizing the internucleosome interactions being measured here. In addition, the amount of DNA released

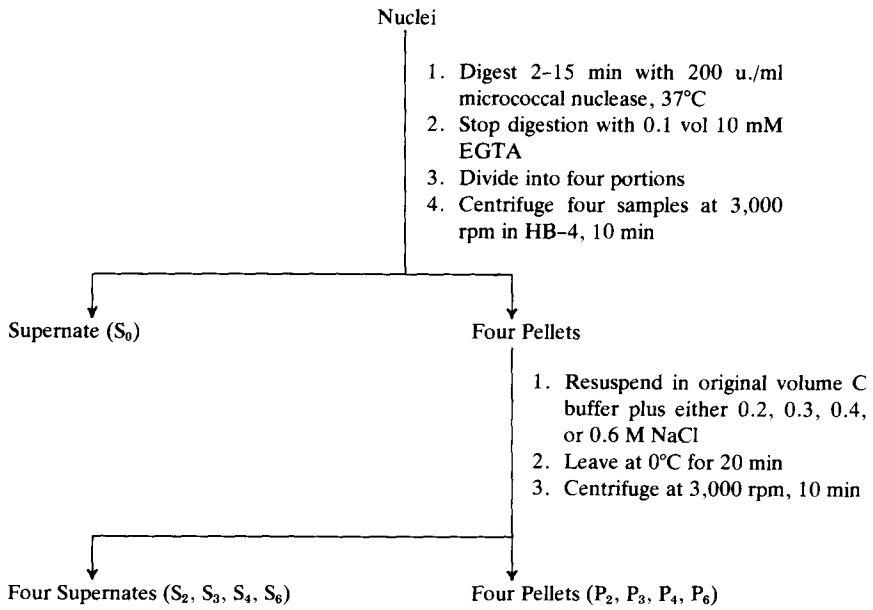

FIGURE 4 Experimental procedure for determining nucleosome release from digested nuclei as a function of increasing NaCl concentrations.

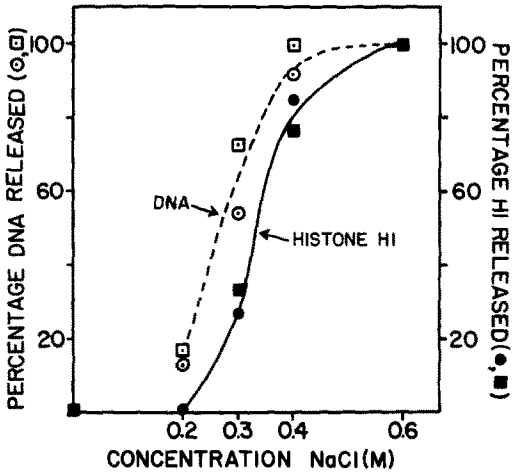

FIGURE 5 Release of nucleosomes and H1 from micrococcal nuclease-digested nuclei as a function of NaCl concentration. The DNA and histone H1 content of supernatant fractions S_0, S_2, S_3, S_4, and S_6, prepared as diagrammed in Fig. 4, were determined, expressed as percentages of the total amount in the digest and plotted against NaCl concentration. (○) DNA and (●) H1 released from a 2 min digest (2% solubilization of the DNA); (□) DNA and (■) H1 released from a 10-min digest (15% solubilization of the DNA).

did not change with time between 10 min and 24 h.

The elution of histone H1 lagged behind that of nucleosomes at all extents of digestion (Fig. 5), with very little or no measurable H1 solubilized in 0.2 M NaCl and up to 30% less H1 than nucleosomes solubilized with 0.3 and 0.4 M NaCl. All the H1 was eluted by 0.6 M NaCl. This indicates either that the stoichiometry of H1 association with nucleosomes is not constant throughout the nucleus or that H1 is associated with and has a higher affinity for some nuclear component in addition to nucleosomes. The stoichiometry of H1 association with the eluted nucleosomes is further considered at a later point in this paper.

Previous work has shown that histone H1 is dissociated from chromatin and nuclei between 0.35 and 0.6 M NaCl (8, 41, 55). In light of the implication that H1 mediates chromatin fiber crosslinking or packing (7, 8, 12, 17, 31, 36, 44, 48), it seemed possible that monomer nucleosomes released by 0.3 M NaCl might exist as dimers or oligomers in solution due to H1 crosslinking. The sedimentation velocity of nucleosomes eluted at each of the NaCl concentrations was therefore investigated in a digest containing only 140–185 base pair fragments of DNA (Fig. 6). The nucleosomes released by 0.2, 0.3, and 0.6 M NaCl all sediment at about 10 S. Thus, internucleosome crosslinks mediated by histone H1 in

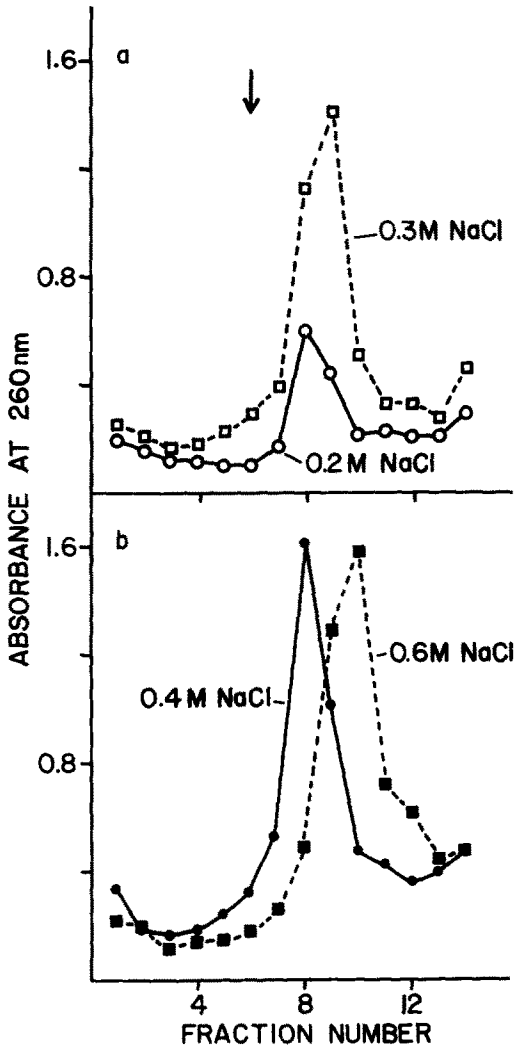

FIGURE 6 Sedimentation properties of monomeric nucleosomes eluted at different NaCl concentrations. 0.2-ml samples of S_2, S_3, S_4, and S_6 prepared as in Fig. 4 from a 10-min micrococcal nuclease digest were applied to 11-ml 10–30% sucrose gradients prepared, respectively, in C buffer plus 0.2, 0.3, 0.4, and 0.6 M NaCl and centrifuged in a Spinco SW 41 rotor at 35,000 rpm for 18 h at 4°C. Absorbance at 260 nm of 0.8-ml fractions collected from the bottom of the tube was determined. Sedimentation was from right to left. (○—○) S_2 in C buffer plus 0.2 M NaCl; (□—□) S_3 in C buffer plus 0.3 M NaCl; (●—●) S_4 in C buffer plus 0.4 M NaCl; (■—■) S_6 in C buffer plus 0.6 M NaCl. The arrow indicates where nucleosome dimers, if present, would sediment under these conditions.

the soluble population of nucleosomes are not detected under these conditions.

Analysis of the Composition of Nucleosomes Eluted at Different NaCl Concentrations

The elution of nucleosomes over a range of NaCl concentrations suggested that the subfractions might differ in structure or macromolecular composition, so the DNA, histone and nonhistone chromosomal protein content of each of the fractions was analyzed by electrophoretic techniques. The compositional differences were easier to analyze in nucleosome fractions that were eluted stepwise with increasing NaCl as diagrammed in Fig. 7. The pattern of DNA and H1 release with the stepwise procedure was indistinguishable from that shown in Fig. 5.

DNA was purified from each of the stepwise-released subfractions from a 2-min micrococcal nuclease digest (2% acid solubilization of the DNA). The size of the fragments was determined by electrophoresis on a 6% acrylamide slab gel (32) (Fig. 8). The fraction eluted by 0.2 M NaCl (SS_2) represented 13% of the total DNA present and consisted almost entirely of monomer and dimer fragments. The average size of the DNA fragments released increased with each successively higher NaCl concentration until the SS_6 fraction (8% of the total digest DNA) which contained very little monomer DNA and a preponderance of fragments larger than octamers. Other subtle differences in the size classes of DNA present in each subfraction were consistently noted. Monomer and dimer fragments in SS_2 were slightly smaller than those in the total digest; monomer fragments in SS_3 were larger than those in SS_2; monomer, dimer, trimer, and tetramer fragments in SS_4 and SS_6 were slightly larger than those in the total. These observations are all consistent with the interpretation that the DNA in the nucleus that is most open and available for micrococcal nuclease attack is the DNA that is eluted at the lowest NaCl concentrations. The size differences between dimers, trimers, and tetramers seem to be more consistent with the gradual shortening of fragments as digestion progresses (33) than with differences in the nucleosome repeat length (15, 37, 40).

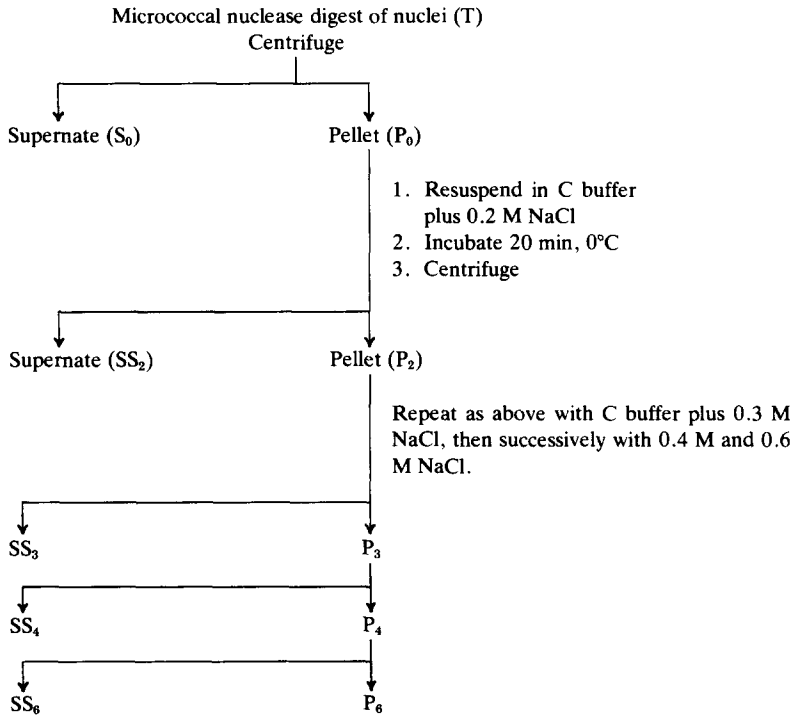

FIGURE 7 Experimental procedure for the stepwise isolation of nucleosomes released from digested nuclei by each successively higher NaCl concentration. The conditions of digestion and centrifugation were as in Fig. 4.

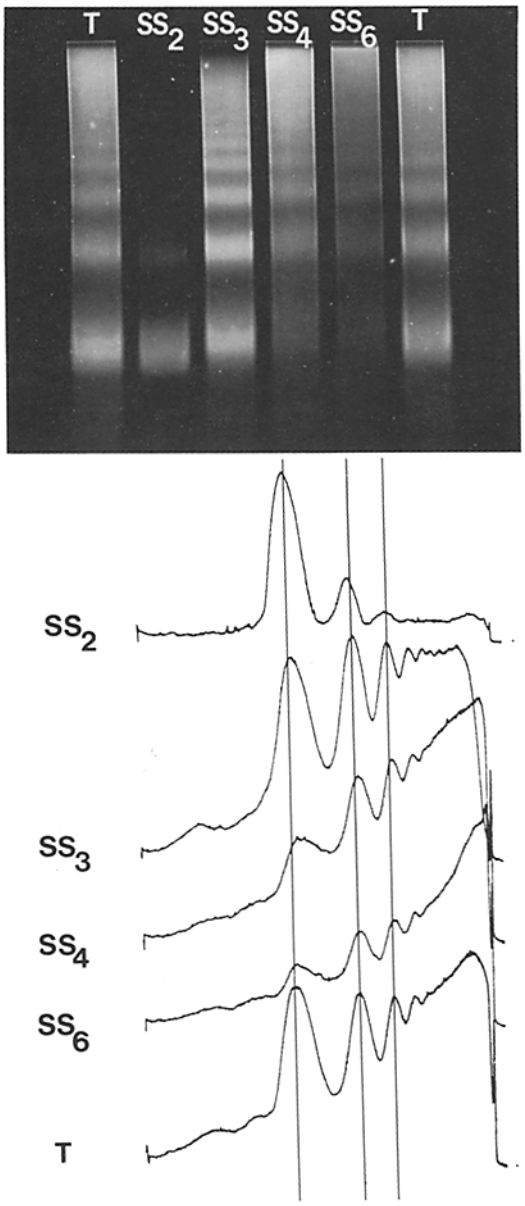

FIGURE 8 Size analysis of the DNA fragments released from digested nuclei by stepwise increases in NaCl concentration. DNA extracted from the stepwise eluted fractions SS_2, SS_3, SS_4, and SS_6 was compared with DNA extracted from the total digest (T) by electrophoresis on a 6% acrylamide slab gel (32). 10 μg of DNA was applied in each case except SS_2 where 5 μg was applied. The gel was stained with ethidium bromide and photographed with UV light. Densitometer tracings of the negative are shown below the gel.

The periodic repeats are sharp in the SS_3 subfraction (41% of the digest DNA) but the periodicity becomes less clear in the SS_4 and SS_6 material. This is particularly noticeable as a decrease in the peak-to-valley optical density change between dimers and trimers on the tracing of this gel (Fig. 8 b). This could mean that the SS_4 and SS_6 DNA fractions contain a minor component with a different nucleosome repeat, that the spacing of nucleosomes in the chromatin eluted by 0.4 and 0.6 M NaCl is not so regular as it is in the SS_3 fraction, or that some of the DNA is not organized in conventional nucleosomes in the SS_4 and SS_6 material.

The total protein content of each of the subfractions from the stepwise elution procedure (Fig. 7) was analyzed by discontinuous SDS acrylamide slab gel electrophoresis (25). Fig. 9 shows, from left to right, the proteins present in the total digest (T), each pellet fraction after treatment with successively higher NaCl concentrations (P_0–P_6)

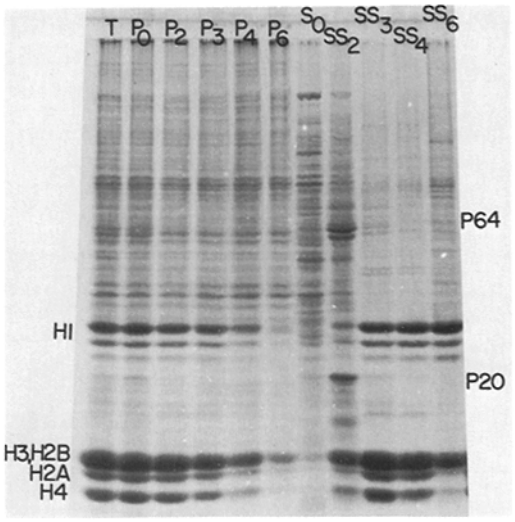

FIGURE 9 Total proteins present in released and residual fractions of digested nuclei treated with increasing NaCl concentrations. The protein content of identical volumes of T and P_0–P_6 prepared as diagrammed in Fig. 7 was compared in the six leftmost tracks of a 12.5% discontinuous SDS acrylamide slab gel (25). The tracks to the right contained respectively protein from four times that volume of S_0 and from volumes of SS_2–SS_6 containing 0.16 A_{260} units. The decreased amounts of nucleosome histones in SS_2 and SS_6 relative to SS_3 reflect the presence of 260 nm absorbing material other than DNA in these fractions. Proteins in S_0 and SS_2 were derived from the same volume of those fractions.

and the proteins eluted with each stepwise NaCl treatment (S_0-SS_6). The protein composition of the total and P_0–P_6 samples is complex; these samples do show clearly, however, the successive disappearance of nucleosome histones and histone H1. The residue in P_6 contains prominent protein species typical of the residual nuclear membrane described by Berezney and Coffey (5) which includes the nuclear matrix with membrane still attached.

The protein composition of the S_0 fraction is also complex. This contains soluble nuclear proteins and no H1 or nucleosome histones, consistent with the previous observation (Figs. 3 and 5) that no DNA was released by EGTA-arrested digestion with micrococcal nuclease. SS_2 contains nucleosome core histones, a trace of H1, a prominent protein of 20,000 daltons apparent molecular weight (P20) and a large number of proteins in the 30–90,000-dalton mol wt range. In contrast, SS_3 and SS_4 contain histone H1, a few nonhistone proteins and no P20. Small amounts of a large number of proteins are present in SS_6. The amount of H1 increases from SS_3 to SS_6 as does the amount of a protein that migrates ahead of H1 with an apparent molecular weight of 26,000 daltons (P26).

The histones and acid-soluble proteins were extracted from each of the subfractions and were compared using two electrophoresis systems. Discontinuous SDS acrylamide slab gel electrophoresis (Fig. 10) showed that SS_2 contains only a trace of histone H1 and major complement of P20. SS_3, Ss_4 and SS_6 have no P20 but contain increasing amounts of H1 relative to H4 (or to the mixture of H3, H2A and H2B) in the normalized ratios of 1.0 for SS_3, 1.5 for SS_4 and 1.8 for SS_6. In addition, the amount of P26 increased from SS_3 to SS_6 as noted above (Fig. 9).

Two-dimensional electrophoresis using the triton-urea acrylamide system (1, 18, 14) in the first dimension and discontinuous SDS acrylamide electrophoresis in the second confirmed the unequal distribution of P20, H1, and P26 (Fig. 11). In addition, this showed that the subspecies of H3 resolved in the first dimension of this system, H3.2 and H3.3 (18), are not distributed in the same relative amounts in SS_2 and SS_6. The H3 in SS_2 is almost entirely H3.2 while both H3.2 and H3.3 are present in SS_6. SS_3 and SS_4 have H3 distributions indistinguishable from SS_6 (data not shown).

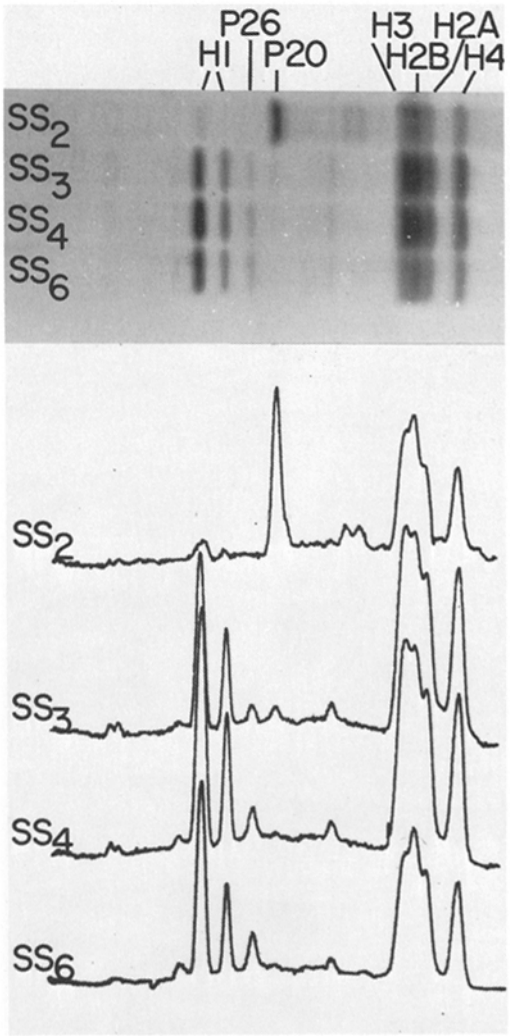

FIGURE 10 Acid-soluble proteins present in the stepwise eluted nucleosome fractions compared by molecular weight criteria. Acid-extracted proteins from SS_2, SS_3, SS_4, and SS_6 were electrophoresed on 12.5% discontinuous SDS acrylamide slab gels (25). Electrophoresis was from left to right. The upper portion of the gel contained no bands and is not shown. Densitometer tracings of the negative are shown below the photograph.

To determine which of the eluted proteins were nucleosome bound, the nucleosomes from each of the stepwise-eluted subfractions were purified by centrifugation through 10–30% sucrose gradients prepared in C buffer containing the NaCl concentration that had been used to solubilize that particular subfraction. The protein content of the nucleosome-containing fractions from the gradients

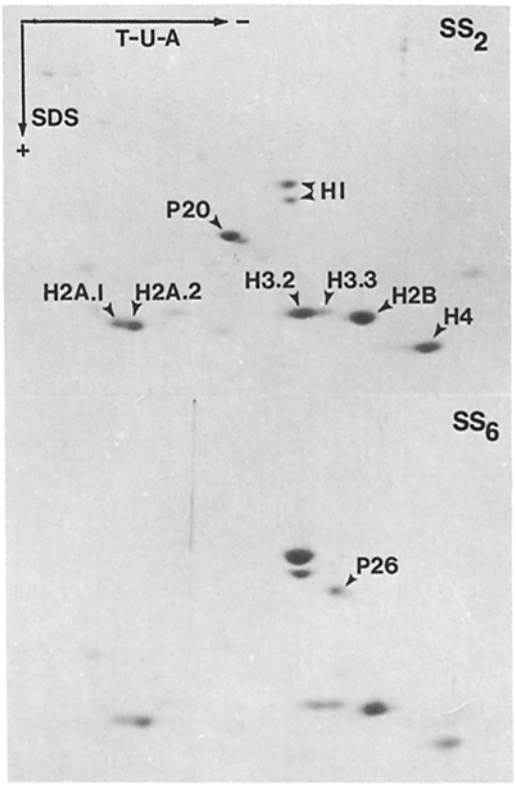

FIGURE 11 Acid-soluble proteins present in the SS_2 and SS_6 nucleosome fractions compared by Triton-urea-acetic acid/discontinuous SDS acrylamide two-dimensional gel electrophoresis. Acid-extracted proteins from SS_2 and SS_6 nucleosome fractions were applied in 10 μl to slab gels prepared as described by Cohen et al. (14) and Alfageme et al. (1). The tracks were cut out and electrophoresed in the second dimension on discontinuous SDS acrylamide slab gels (25). The histone species are identified using the nomenclature of Franklin and Zweidler (18).

was analyzed by electrophoresis on discontinuous SDS-acrylamide slab gels (Fig. 12). Minor amounts of several nonhistone proteins cosedimented with nucleosomes in all the subfractions including species with apparent molecular weights of 18, 19, and 44,000 daltons. The SS_2 fraction in addition contained a prominent protein at 64,000 daltons (P64) and other minor species in the molecular weight range of 30–70,000 daltons. To test the possibility that these proteins appeared only in the SS_2 fraction because they eluted between 0.2 and 0.3 M NaCl, the SS_2 material was also centrifuged through a gradient prepared in 0.3 M NaCl (sample SS_2', Fig. 12). None of the SS_2 nucleosome-bound proteins was removed by 0.3 M NaCl (or by 0.6 M NaCl [data not shown]). The prominent acid-soluble protein P20 that was solubilized with SS_2 but not present in S_0 (Figs. 9, 10, and 11) is not bound to the SS_2 nucleosomes at this ionic strength. The SS_3 fraction had H1 associated with the nucleosomes but the SS_4 and SS_6 nucleosomes did not, indicating that H1 is completely removed from nucleosomes between 0.3 and 0.4 M NaCl. Small amounts of a number of proteins in the molecular weight range of 64,000–100,000-daltons were bound to the SS_6 subfraction.

The data shown in Fig. 12 are from the same 2-min micrococcal nuclease digest that was used for

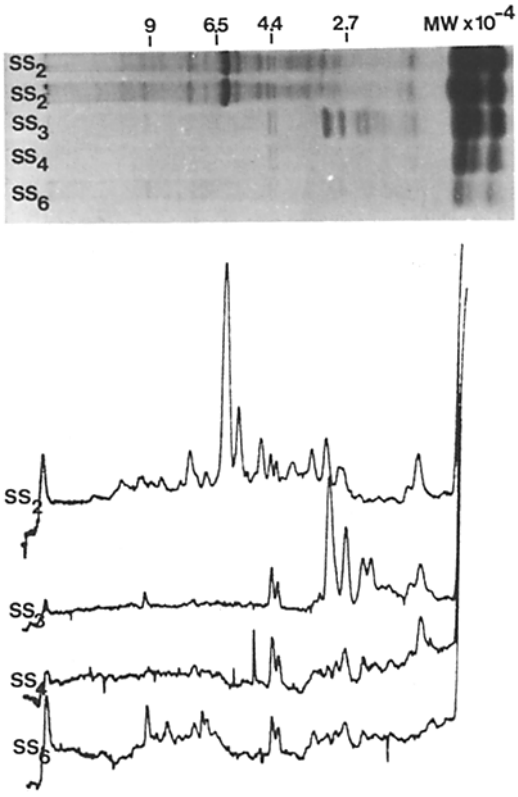

FIGURE 12 Nucleosome-bound proteins present in subfractions released from digested nuclei treated stepwise with increasing NaCl concentrations. Nucleosomes from SS_2, SS_3, SS_4, and SS_6 prepared as in Fig. 7 were purified on sucrose gradients as in Fig. 6. In addition, SS_2 was centrifuged in a gradient prepared in C buffer plus 0.3 M NaCl (SS_2'). The proteins complexed with 20 μg of DNA in the purified nucleosome fractions were compared by electrophoresis on 12.5% discontinuous acrylamide SDS slab gels (25). Electrophoresis was from left to right. Densitometer tracings of the negative are shown below the gel.

Fig. 8. In other experiments, the length of time of digestion was varied and the protein content of every fraction from similar gradients was determined by SDS-acrylamide gel electrophoresis. At all extents of digestion, the proteins shown in Fig. 12 cosedimented with the DNA peak (data not shown). These data are consistent with results previously reported (50) which showed that the same spectrum of nonhistone proteins remained nucleosome associated during reversible divalent cation-dependent aggregation of rat liver monosomes prepared by gel filtration technics. It therefore seems likely that these proteins are nucleosome-bound.

Thus, macromolecular composition of the nucleosome subfractions eluted by increasing concentrations of NaCl differed significantly from one fraction to the next. SS_2 contained the smallest DNA fragments but no histone H1, was depleted in a subspecies of histone H3, contained a prominent nucleosome-bound protein P64, and was solubilized with an acid-soluble protein, P20. SS_3 was released with H1 bound and contained few nonhistone proteins. SS_4 and SS_6 contained the largest DNA fragments, were solubilized with increasing amounts of histone H1 which was not nucleosome bound at these salt concentrations, and also contained increasing amounts of a protein, P26.

DISCUSSION

This work describes biochemical properties of nucleosomes related to the internucleosome packing forces present in micrococcal nuclease-digested rat liver nuclei. The nuclei were isolated under ionic conditions that were optimized for retention of native chromatin morphology (11). The digestion was arrested by selectively chelating the Ca^{2+} required for micrococcal nuclease activity with EGTA, a procedure that does not substantially alter the divalent cation-dependent morphological differences in extent of chromatin condensation in the nuclei when Mg^{2+} is also present. In addition, no free nucleosomes were released from the EGTA-arrested digests, consistent with the interpretation that the forces responsible for packing nucleosomes in the nucleus are not measurably disturbed by this procedure. These criteria do not prove that internucleosome packing forces are native after EGTA-arrested digestion; however, in contrast with the loss of morphological differentiation in chromatin condensation and partial release of nucleosomes observed with EDTA arrest of digestion, the EGTA procedure seems preferable for experiments in which the native compostiion or interactions of nucleosomes may be important.

Perturbation of the digested nuclei with increasing concentrations of NaCl, a procedure known to gradually eliminate differences in the extent of chromatin condensation in intact nuclei (29, 43), resulted in the gradual release of nucleosomes and H1 in a noncoordinate manner. The compositional differences between nucleosomes released at different salt concentrations show that the procedure described here does distinguish between structurally different chromatin substructures, suggesting that the nucleosome subfractions derive from functionally different parts of the nucleus. Furthermore, this fractionation accounts for all the DNA in the digested nuclei.

Comparison of the fragment size of the DNA associated with the nucleosome populations released at each successively higher NaCl concentration in a digest where 1–2% of the DNA had been rendered acid soluble showed that the most extensively digested material was released at 0.2 M NaCl and that the largest fragments were solubilized at 0.6 M NaCl. Thus, the nucleosomes released by 0.2 M NaCl must be those structures that are most open and available for nuclease attack. It is important to realize that the pattern of DNA release from nuclei with increasing NaCl did not change significantly over the range of 2–20% digestion of the DNA, showing that the differential salt elution is gradually dissociating packing interactions that are not measurably affected by extent of digestion.

The limited digestion of nuclei with DNase I and DNase II has shown that the actively transcribing genes are attacked preferentially by these enzymes (19, 21, 22, 26, 27, 53), suggesting that this fraction of the chromatin has structural properties different from those of bulk. Studies with micrococcal nuclease have shown that both transcriptionally active and inactive regions of the genome are organized in the nucleosome structure (3, 6, 24, 46). But evidence has been presented that satellite DNA in the kangaroo rat is digested at a slower rate by this enzyme (6). In addition, in digests of oviduct nuclei and ovalbumin genes are enriched in the smallest micrococcal nuclease-generated fragments (4), indicating that the transcriptionally active structures are more available to this enzyme as well. The smaller than average size of the DNA fragments in the nucleosome subfraction

released by 0.2 M NaCl in the experiments reported here thus suggests that the SS_2 material is derived from transcriptionally active chromatin. Circumstantial support for this concept comes from comparing the fraction of DNA released in SS_2 (13–17%) with the calculation by Davidson et al. in the appendix to Gottesfeld et al. (20) that the template active fraction constitutes 20 ± 3% of the rat liver genome. The figures agree well.

In addition to smaller DNA fragment size, the SS_2 nucleosome subfraction differs from the material released at higher NaCl concentrations in other properties. Very little histone H1 is solubilized with SS_2: however, a substantial amount of a smaller acid-soluble protein of apparent molecular weight of 20,000 daltons (P20) is completely solubilized by 0.2 M NaCl. P20 is not nucleosome-bound in 0.2 M NaCl. Nevertheless, the absence of P20 in soluble nuclear protein fraction S_0 and the coordinate release of the SS_2 nucleosomes with P20 suggests that P20 may be the packing protein mediating internucleosome interactions between SS_2 nucleosomes, analogous with the role of H1 in packing SS_4 and SS_6 nucleosomes. One reason for proposing this is that an acid-soluble protein with similar properties (originally known as component T and now called H6) is chromatin-bound in trout testis. It is extracted by 0.3 M NaCl (34, 54) and has been shown to be released from trout testis nuclei during DNase I digestion of actively transcribing genes and to be enriched in the "active" chromatin prepared after DNase II digestion (28). It is possible that P20 is the rat liver protein analogous to H6.

The absence of histone H1 in the SS_2 fraction does not exclude the possibility that H1 plays a role in mediating the packing of SS_2 nucleosomes in the nucleus. It is possible that H1 does interact with SS_2 nucleosomes but also interacts with a higher affinity with some other nuclear structure or, alternatively, that the H1 interaction site on SS_2 nucleosomes (or between them) is eliminated by the digestion procedure. The experiments reported here do not answer these questions; however, the data do indicate that the interaction, if any, of SS_2 nucleosomes with H1 is not the same as the H1-nucleosome interactions in the other subfractions.

The SS_2 nucleosomes contain several bound nonhistone chromosomal proteins that are not present in the other nucleosome fractions, including a prominent protein of an apparent molecular weight of 64,000 daltons (P64) which, staining intensity indicates, must be present in a substantial portion of the SS_2 nucleosomes. In addition, SS_2 is depleted in the H3.3 component of histone H3 as shown by two-dimensional acrylamide gel electrophoresis. These protein compositional differences, the increased availability of the nucleosomes extracted by 0.2 M NaCl to micrococcal nuclease attack, and the possible association of this fraction with P20 instead of histone H1 all point to the conclusion that SS_2 derives from a part of the nucleus that is involved in a special function. The possibility that this function is transcription is currently being tested.

The material eluted from the digested nuclei by 0.3–0.6 M NaCl includes about 85% of the nucleosomes and all of the histone H1. The solubilization of H1 is not parallel with the release of these nucleosomes. This is apparent from measurements of total H1 solubilized at a given NaCl concentration, as shown in Fig. 5, and from the increasing amounts of H1 present in stepwise eluted fractions SS_3, SS_4, and SS_6 relative to both DNA content (Fig. 9) and to concentration of the other nucleosome histones (Fig. 10). The packing forces between SS_3 nucleosomes must be different from those between SS_4 and SS_6 nucleosomes because SS_3 is released from digested nuclei with some histone H1 bound, while SS_4 and SS_6 can only be solubilized when H1 is completely dissociated. This difference was independent of extent of nuclease digestion (Fig. 5), and it implies that at least two types of H1-mediated nucleosome packing can exist that differ in both stoichiometry of H1 association and strength of the packing interaction.

These differences in stoichiometry of H1 association with nucleosomes are probably not related to the cooperativity of H1 binding to the nucleosomes and DNA based on the length of the DNA strand (47, 48). In the experiments reported here, the non-parallel solubilization of H1 and nucleosomes occurred both in partial digests and in digests that were carried to the monomeric nucleosome stage so that they could not be explained by a rearrangement of histone H1 after digestion due to a preference for longer nucleosome strands. Other workers have reported that H1 may rearrange at higher salt concentrations (10, 13); however, these observations have all been made on chromatin prepared at low ionic strength which Renz et al. (48) have shown destabilizes and randomizes H1 association with nucleosomes. It seems likely, therefore, that the results reported

here reflect either the native state of histone H1 in the nucleus or a preference for H1 association with particular nucleosome structures.

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