Circular Dichroism, Thermal Denaturation, and Deoxyribonuclease I Digestion Studies of Nucleosomes Highly Enriched in High Mobility Group Proteins HMG 1 and HMG 2†

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ABSTRACT: Salt-soluble (S) nucleosomes that contain near equimolar high mobility group nonhistone chromosomal proteins HMG 1 and HMG 2 and lack histone H1 were isolated from mouse myeloma nuclei. Comparisons of the sedimentation, near-UV circular dichroism, thermal denaturation, and pattern of DNase I digestion of S nucleosomes with these properties of nucleosome cores or "typical" nucleosomes containing H1 did not detect significant differences. These results indicate that HMG 1 and 2 do not affect the conformation and stability of nucleosomes or nucleosomal DNA and are consistent with the proposal that major functions of HMG 1 and 2 are to replace H1 and maintain the (micro) solubility and accessibility of local chromatin regions. In contrast to these similarities, the initial rate of DNase I digestion of S nucleosomes was ~3 times that of chromatin depleted in S nucleosomes. This is consistent with a relation of S nucleosomes to transcription and suggests that subtle factors (not necessarily HMG 1 and 2) determine DNase I susceptibility.

We have recently described the isolation of an unusual subset of nucleosomes that are released from mouse myeloma nuclei under near-physiological ionic conditions after very slight treatment with micrococcal nuclease (Jackson et al., 1979). These nucleosomes contain an apparently normal complement of core histones (H2a, H2b, H3, H4) and ca. 200 bp[1] length DNA, but lack histone H1, and instead contain near-stoichiometric amounts of two high mobility group nonhistone chromosomal proteins, HMG 1 and HMG 2. They are also enriched in lesser amounts of several other nonhistone proteins.

HMG 1 and HMG 2 are relatively abundant nonhistone proteins (one copy per 5–10 nucleosomes) and therefore are likely to be structural, rather than regulatory, components of chromatin (Johns et al., 1975). Studies correlating the release of specific proteins with the preferential degradation of active genes by DNase I and DNase II have suggested that HMG 1 and HMG 2 (and the similar protein, HMG-T, of trout testis) are associated with transcriptionally active chromatin (Levy et al., 1977, 1979). In addition, brief digestion of oviduct and trout testis nuclei with micrococcal nuclease yields nucleosome length DNA that is highly enriched in transcribed sequences (Bellard et al., 1978; Bloom & Anderson, 1978; Levy et al., 1979; Levy & Dixon, 1978). The fact that nucleosomes containing HMG 1 and 2 are isolated from myeloma chromatin under very similar conditions and represent the majority of nucleosomes released (≥70%: S. Chambers and R. L. Rill, unpublished observations) also suggests an origin from active chromatin. The possibility that HMG 1 and 2 function in the higher order organization of chromatin has also been suggested (Matthew et al., 1979).

We presume that the nucleosomes containing HMG proteins are functionally distinctive in some way. Replacement of H1 by HMG 1 and 2 renders nucleosomes soluble under near-physiological conditions. This phenomenon could entirely explain the function of these proteins, namely, to maintain a portion of chromatin in a (micro) soluble and accessible state. Alternatively, HMG 1 and 2 could function by causing a major conformational change in the nucleosome core, e.g., to facilitate readthrough of nucleosome DNA during transcription, replication, or other processes.

To distinquish between these possibilities we have compared the sedimentation, circular dichroism, thermal denaturation, and DNase I digestion of nucleosomes containing and lacking HMG 1 and 2. These methods are known to be sensitive indicators of various aspects of the DNA and histone conformations within nucleosomes (Rill et al., 1978; Rill, 1979). No significant effects of the presence of HMG 1 and 2 on the nucleosome structure could be discerned by the above methods, although the initial *rate* of DNase I digestion of nucleosomes containing HMGs was nearly 3 times that of whole chromatin.

Materials and Methods

Isolation of S and P Nucleosomes. Nuclei were obtained from myeloma line 66-2 tumors grown in BALB/C mice. Methods for preparing nuclei and soluble (S) chromatin enriched in HMG 1 and HMG 2 were as described previously (Jackson et al., 1979). Briefly, the procedure involves incubation of nuclei with micrococcal nuclease (Worthington) at 0 °C followed by dialysis against saline solution (120 mM KCl, 30 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM PMSF, and 15 mM Tris, adjusted to a final pH of 7.5). The supernants and pellets obtained after final sedimentation of nuclei are referred to as the S and P fractions, respectively.

Soluble mononucleosomes, the major S fraction component, were isolated by sedimentation on linear 5–20% sucrose gradients for 11 h at 40000 rpm in an International SN-283 rotor maintained at 4 °C.

Typical nucleosomes (containing H1) and nucleosome cores were obtained by redigestion of the P fraction nuclei with micrococcal nuclease, followed by sedimentation as above.

Electrophoresis of proteins was performed on 15-cm slab gels of 18% polyacrylamide containing $NaDodSO_4$ (Jackson

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[1] Abbreviations used: bp, base pairs; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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et al., 1979). Staining was with 0.25% Coomassie blue R250 (Bio-Rad) or with Uniblue A (Eastman) in methanol–acetic acid–water (50:10:40). Gels were scanned in a Gelman DCD-16 recording densitometer at 580 nm.

Electrophoresis of native DNA was performed on 6% polyacrylamide gels (Jackson et al., 1979). Denatured DNA was electrophoresed on 12% gels containing 7 M urea (Maniatis et al., 1975).

DNase I digestion of mononucleosomes or total P chromatin was done at 37 °C in 10 mM Tris-HCl (pH 7.5), 0.75 mM $MgCl_2$, 0.1 mM EGTA, and 0.1 mM cacodylic acid with 1 μg of DNase I (Worthington) per 50 μg of DNA. EGTA was added to chelate Ca^{2+} and prevent digestion by any residual micrococcal nuclease. The $MgCl_2$ concentration was kept as low as possible to minimize precipitation of chromatin and nucleosomes containing H1. Digestion was terminated by addition of EDTA to 5 mM, and DNA was extracted by the method of Britten et al. (1974).

Thermal denaturation was monitored with a Beckman Acta CII spectrophotometer. A thermistor probe and thermivolt thermometer (Yellow Springs Instruments) provided a continuous record of absorbance at 260 nm vs. temperature on an X-Y recorder (Houston Instruments). The temperature was programmed to increase at 1 °C/min by using a Neslab TP-2 temperature programmer and a circulator bath. Degassed solutions were overlayered with buffer-extracted mineral oil and cuvettes were sealed with Teflon stoppers. Derivative melting curves were computed from data taken at 1 °C intervals, with corrections for the thermal expansion of water. Samples were exhaustively dialyzed against 0.25 mM EDTA and 0.1 mM PMSF (pH adjusted to 7.0).

Circular dichroism spectra were obtained on a Jasco ORD/UV-5 recorder with a CD attachment and a Sproul Scientific SS20 modification. Molecular ellipticities were calculated on the basis of phosphate molarities determined from the absorbance at 258 nm (measured with a Cary 15 spectrophotometer). Samples were dialyzed exhaustively against 5 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, 0.1 mM cacodylic acid, and 0.1 mM PMSF.

Results

Isolation of S and P Fraction Mononucleosomes. Figure 1 shows typical density gradient profiles of S fraction and redigested P fraction nucleoproteins sedimented in the same rotor under identical conditions. No significant differences in sedimentation coefficients of S and P mononucleosomes (~11 S) or dinucleosomes were evident in this and several similar experiments.

Preparations of S mononucleosomes routinely contained all four core histones in similar amounts, HMG 1 and 2 in near stoichiometric amounts, and small amounts of other nonhistone proteins (Figure 2). H1 was present in trace amounts, if at all. DNA from S nucleosomes migrated as a broad band with a median size of 190–200 bp and range from ~140 to ~230 bp [e.g., see Figure 5 and Jackson et al. (1979)]. Electrophoresis of S nucleoproteins has shown that S nucleosomes from gradients are contaminated with small amounts of nucleosome cores lacking HMG proteins (Jackson et al., 1979). For these experiments digestion was controlled so that only 3–5% of the total chromatin DNA was solubilized and less than about 10% of the ~11S gradient fractions were nucleosome cores.

Nucleosomes from P fractions contained the usual complement of core histones and variable amounts of H1, depending on the extent of redigestion. Samples used for CD and DNase I digestion contained significant amounts of H1

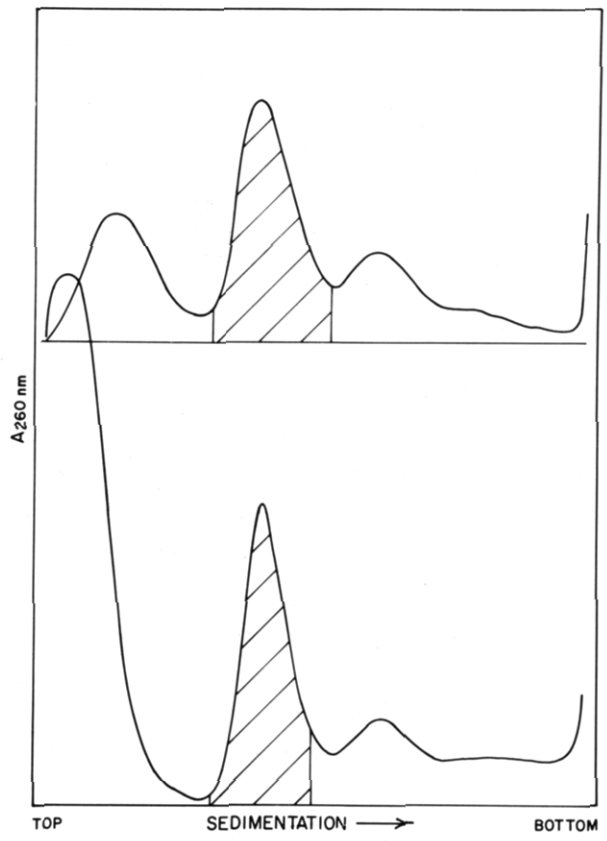

FIGURE 1: Sedimentation pattern of total S fraction chromatin (top) and redigested P fraction (bottom) on 5–20% linear sucrose gradients. Mononucleosome (~11 S) fractions were pooled as indicated.

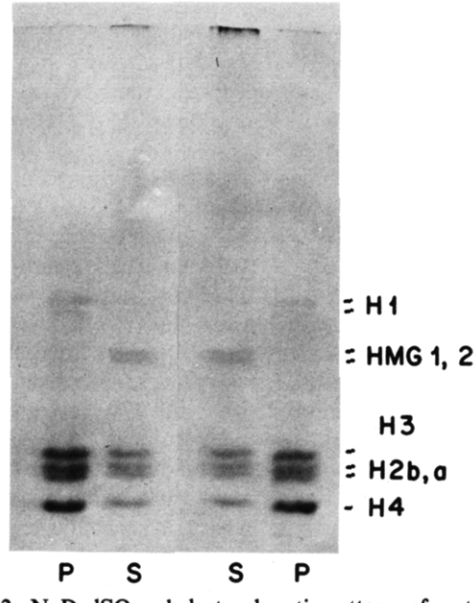

FIGURE 2: $NaDodSO_4$ gel electrophoretic patterns of proteins from S nucleosomes and P nucleosomes.

and DNA near 200 bp long, while those thermally denatured were mainly cores lacking H1 (figure 2). These variations do not affect the major conclusions of the studies.

Circular Dichroism. The positive CD spectrum of DNA from 260 to 300 nm characteristic of the B form is suppressed to less than half of its normal intensity in typical chromatin preparations, and even more so in nucleosome cores (Rill, 1979; Fasman, 1978). Since histones do not contribute in this spectral region, this phenomenon can be attributed to the unique geometry and/or environment of DNA within nucleosomes. Recent studies suggest that the magnitude of the

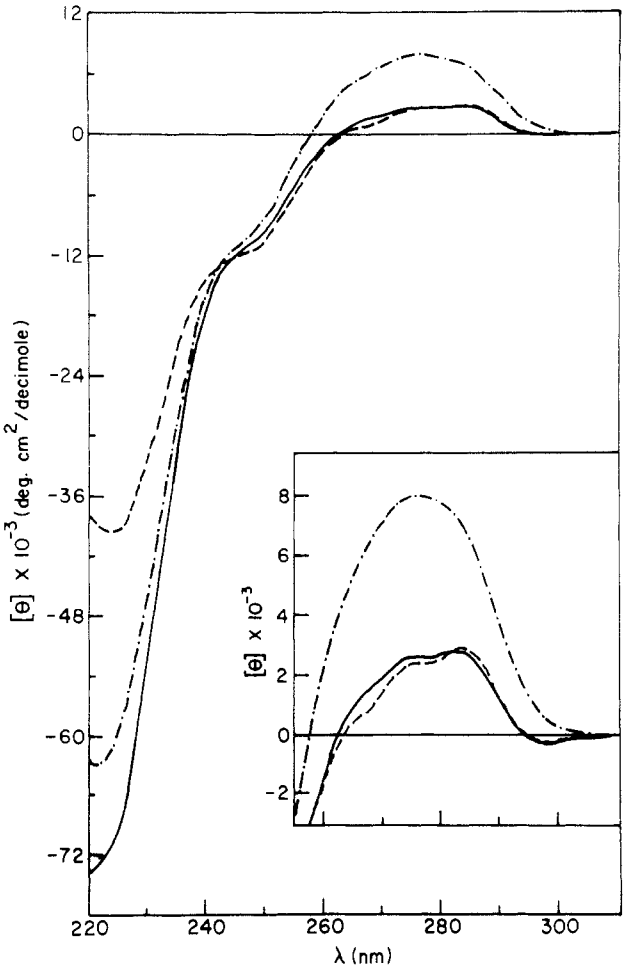

FIGURE 3: Circular dichroism spectra of S mononucleosomes (—), P mononucleosomes (---), and S nucleosomes plus 0.2% $NaDodSO_4$ (------). Inset: Same spectra from 250 to 310 nm, expanded scale. Ellipticities calculated on the basis of moles DNA phosphate.

274-nm band can be interpreted directly in terms of the average rotation per base pair (Baase & Johnson, 1979).

CD spectra of S and P nucleosomes containing HMG and H1, respectively, and similar DNA lengths are compared in Figure 3. Both spectra are strongly suppressed compared to that of DNA (obtained by adding $NaDodSO_4$ to S nucleosomes), with positive bands at 274 nm and 283 nm, and a weak negative band at 298 nm, characteristic of nucleosome preparations (Baase & Johnson, 1979; Rill, 1979; Fasman, 1978). These spectra are essentially identical from 260 to 310 nm, within the error limits of our instrument.

The spectrum below 240 nm, with a strong negative band at 220 nm, is mainly due to α-helical regions of proteins. The intensity of the P nucleosome band ($\theta_{220} = -39 \times 10^3$ deg cm^2/dimol of phosphate) is in good agreement with other reports and has been interpreted to indicate a helix content of core histones of 45–50% (Fasman, 1978; Thomas et al., 1977). A much larger band was observed for S nucleosomes ($\theta_{220} = -74 \times 10^3$ deg cm^2/dimol), suggesting that nonhistone proteins bound to these nucleosomes possess high helix contents. This result is consistent with the report that HMG 1 and HMG 2 are 40–50% α-helical in solutions of varying ionic strengths over a broad pH range (pH 4–9) (Cary et al., 1976). An interpretation solely in terms of HMG and histone conformations is not possible because of the presence of other nonhistone proteins.

Thermal Denaturation. Thermal denaturation of chromatin DNA at low ionic strengths is multiphasic, reflecting differing

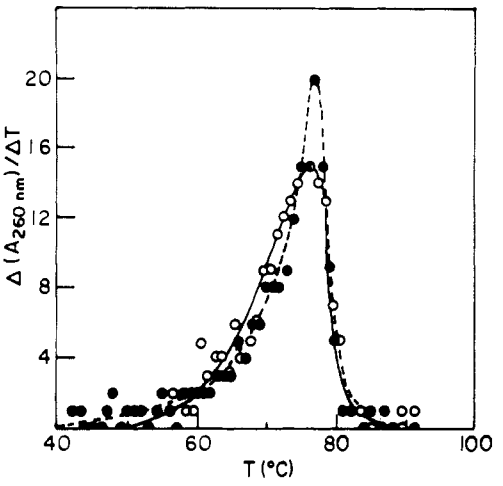

FIGURE 4: Derivative thermal denaturation curves of S mononucleosomes (●) and P nucleosome cores (○).

degrees of stabilization by electrostatic charge neutralization and other interactions. The greatest stabilization occurs within nucleosome cores, demonstrating the strength and dominating influence of core histone binding to DNA (Li & Bonner, 1971; Weischet et al., 1978).

Thermal denaturation curves of S nucleosomes containing HMG proteins and P nucleosome cores were very similar (Figure 4) and agreed well with published data on nucleosome cores (Li & Bonner, 1971; Weischet et al., 1978). Most DNA in both samples denatured over a narrow range, with T_m's of 76 °C (P cores) and 77 °C (S nucleosomes). A slightly larger proportion of S nucleosome DNA denatured at low temperatures (<60 °C), perhaps reflecting an expected difference in the stabilization of linker DNA (Woodcock & Frado, 1975; Weischet et al., 1978). Clearly the presence of HMG 1 and 2 does not significantly alter the degree of histone stabilization of DNA in the nucleosome core, although small differences in the degree of cooperativity of the high-temperature transitions that are difficult to interpret were noted.

DNase I Digestion Specificity. Cleavage of nucleosomes in chromatin and in isolated form by DNase I occurs at discrete sites spaced at 10.4 nucleotide intervals along both strands (Prunell et al., 1979). Restriction of cleavages to these positions reflects the topological accessibility of DNA strands as they simultaneously rotate about the helix axis and wind about the core histone complex. The periodicity of cleavage appears most directly related to the DNA geometry, i.e., the number of residues per helix repeat, while the frequency of cleavage at specific sites with respect to the end of the nucleosome core may reflect the degree of protection by histones (Sollner-Webb et al., 1978). Neither protein-free DNA nor improperly reconstituted histone–DNA complexes are digested at periodic sites.

Electrophoretic patterns of denatured DNA from DNase I treated S and P mononucleosomes are shown in Figure 5. The gel was stained briefly with ethidium bromide and then with Stains-all to increase the sensitivity range. Examination of the Stains-all pattern shows that both samples contained DNA of similar average length, near 200 bp, but that the length distribution in S nucleosomes was broad. Both samples were free of large DNA and contained only traces of DNA smaller than 140 bp. The pattern of small DNA in the whole S fraction and S nucleosomes (most evident with ethidium staining) is similar to that generated by DNase I digestion of chromatin. This suggests that the endogenous DNase of myeloma nuclei noted previously (Jackson et al., 1979) is DNase I like with respect to its cleavage specificity and

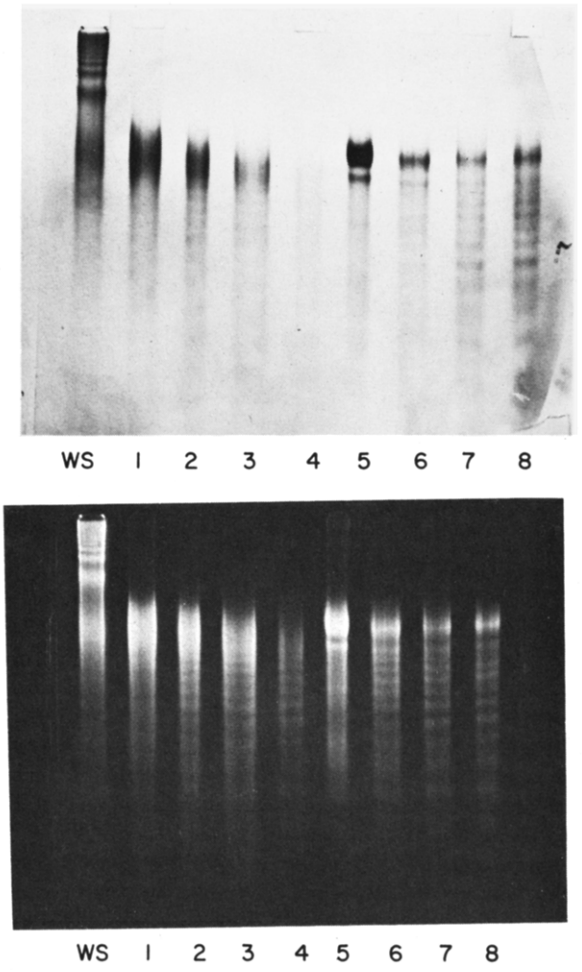

FIGURE 5: Pattern of DNase I digestion of S and P mononucleosomes. Denatured DNA was electrophoresed on gels containing 7 M urea. Left lane (WS) whole (undigested) S fraction; lanes 1–4, S mononucleosomes digested for 0, 6, 18, and 50 min; lanes 5–8, P mononucleosomes digested for 0, 6, 18, and 50 min. Upper photograph was taken after Stains-all staining. Lower photograph was taken of the same gel after ethidium bromide staining. Each sample (1–8) contained the same amount of DNA *prior to* DNase I digestion.

preferentially attacks S nucleosomes. In contrast, the patterns of small DNA fragments from P nucleosomes are less distinct and resemble the pattern expected from micrococcal nuclease digestion.

Both S and P nucleosomes yielded nearly identical ladders of bands at all stages of digestion with DNase I. The patterns differ in degree of background, but not in positions or relative intensities of the bands. The higher background of the S patterns is expected from the broader distribution of initial DNA lengths. Since the full ladder was observed from the least to the most digested sample—which was degraded almost entirely to ethanol-soluble oligonucleotides—digestion of most S nucleosomes must have proceeded through discrete intermediates. The possibility that a small fraction was rapidly digested at random or at different intervals cannot be ruled out. We conclude that the presence of HMG 1 and 2 does not significantly alter the factors determining the specificity of DNase I cleavage of nucleosome cores.

DNase I Digestion Kinetics. Although the specificities of DNase I digestion of S and P nucleosomes appeared identical, differences in the rates of digestion were suggested by the above gel patterns. Identical amounts of both types of nucleosomes were digested for each time period, and all samples were prepared identically for electrophoresis; thus the quantities of DNA in each lane should reflect the digestion rates.

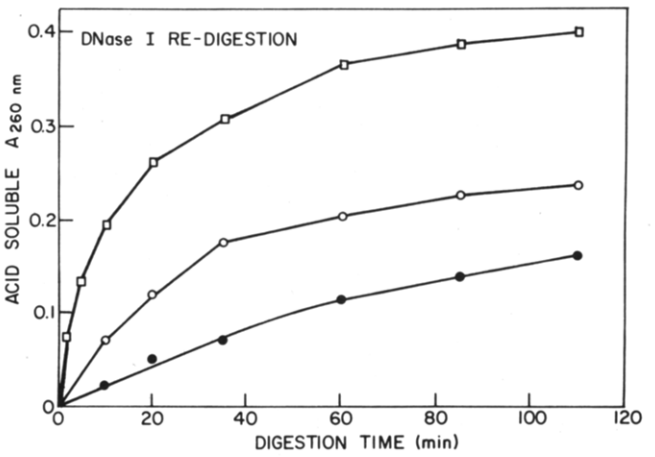

FIGURE 6: Time course of DNase I digestion of S mononucleosomes (○), total P chromatin (●), and protein-free S nucleosome DNA (□). Each sample initially contained the same amount of DNA. An acid-soluble absorbance (A_{260}) of 0.4 is equivalent to ~70% digestion.

The Stains-all pattern shows that most S nucleosome DNA was reduced to less than 140 bp long after 16 min and was almost completely ethanol-soluble after 50 min. In contrast, most P nucleosome DNA remained ethanol insoluble, and much remained larger than 140 bp after digestion for 50 min.

For more precise comparison of the rates of digestion, the time course of acid-soluble oligonucleotide release upon DNase I digestion of a different preparation of S nucleosomes and of the corresponding *total* P fraction (mainly large oligonucleosomes) was carefully measured. In agreement with the impression from the above electrophoretic patterns, the initial rate of digestion of S nucleosomes was approximately 3 times that of whole P chromatin (and about one-fourth that of deproteinized S nucleosome DNA; Figure 6). Evidently S nucleosomes are more susceptible to DNase I degradation than even the most accessible portions of the remaining P chromatin. Since transcribed DNA in chromatin is preferentially degraded by DNase I (Weintraub & Groudine, 1976; Garel & Axel, 1976), the rapid digestion of S nucleosomes strongly supports the proposition that they are related to transcriptionally competent chromatin. This property cannot be attributed only to the presence of HMG 1 and 2 because of other unusual components of S nucleosomes. In particular, recent data have shown that these nucleosomes contain significant amounts of other smaller HMG proteins, an unidentified histonelike protein, and a subpopulation of rapidly acetylated histones (S. C. Chambers, J. B. Jackson, and R. L. Rill, unpublished results). Curiously, none of these factors seem to significantly affect the measured conformational properties of nucleosomes, excepting susceptibility to DNase I.

Discussion

Nucleosomes containing HMG 1 and HMG 2 are preferentially excised from chromatin in situ by micrococcal nuclease and are soluble under ionic conditions that precipitate isolated chromatin and nucleosomes containing H1 or H5. They are also highly enriched in a specific subset of other nonhistone proteins and are depleted in 5-methylcytosine (Jackson et al., 1979). These properties demonstrate an origin from a structurally distinct portion of chromatin containing a subset of DNA sequences. This structural uniqueness must reflect some specific chromatin function(s) and may be related to transcriptional activity, as noted above.

Although the relevance of these nucleosomes to transcription is not fully established, structural studies can provide information about the influence of HGM 1 and 2 on the nucleo-

some conformation and thereby about the possible role of these proteins in functional units of chromatin. The data presented show that under the conditions of our measurements HMG 1 and 2 do not cause significant changes in the overall folded conformation of nucleosomes, the stabilization of DNA by histones, or the conformation and environment of DNA as it wraps about the octameric core histone complex. In this respect they mimic the effect (or lack of effect) of H1 on nucleosome core conformation.

HMG 1 and 2 appear to replaced H1 on nucleosome spacers in certain regions of chromatin (Levy & Dixon, 1978; Levy et al., 1979; Bakayev et al., 1978). We have suggested that one purpose of these replacements may be to unfold higher order (supranucleosomal) chromatin structure and/or increase the accessibility (microsolubility) of functional chromatin (Jackson et al., 1979). This suggestion is based on the well-known ability of H1 to condense chromatin (Renz et al., 1977; Thoma & Koller, 1977; Varshavsky et al., 1977) and the fact that nucleosomes containing HMG 1 and 2 are soluble under near-physiological ionic conditions, while those containing H1 are not. Since HMG 1 and 2 do not markedly affect the conformation of nucleosomes, this solubilization effect remains as their most evident influence on fundamental nucleosome properties. As pointed out previously (Jackson et al., 1979), this effect is consistent with the differences in HMG and H1 amino acid sequences (Walker et al., 1976). Although binding of HMG 1 and 2 may cause subtle conformational changes in nucleosomes, the function(s) of these proteins must be related in an important way to the influence of their highly negative C-terminal sequence regions on the local environment and accessibility of nucleosomes in vivo.

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