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Studies on the stability of the higher-order structure of rat liver chromatin containing high-mobility-group proteins

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The stability of the higher-order structure of chromatin containing high-mobility-group (HMG) proteins has been studied in rat liver nuclei by mild micrococcal nuclease digestion at low temperature and fractionation by sucrose gradient centrifugation. Nuclei preparation and digestion, chromatin solubilization and analysis have been carried out in two ionic conditions, 140 mM and 40 mM monovalent cation concentration, avoiding drastic changes in ionic conditions and temperature during preparation and analysis. During the time course of digestion at 140 mM ionic strength a material stable at 80 S appears, whose DNA is cleaved at values around 12 nucleosomes. The distribution of HMG proteins in different chromatin fractions was analyzed by immunodot using antibodies elicited against proteins HMG-1, HMG-2, and HMG-14 and 17. It appears that these proteins have a distribution distinctly different from the bulk of chromatin. They are never found in the chromatin fragments that keep their internucleosomal interactions, indicating that these proteins tend to accumulate in points where the chromatin has a less stable structure.

Several levels of organization are required to explain the folding of DNA in eukaryotic nuclei. The best-defined and characterized of these structures is the nucleosome but in order to explain the 30-nm chromatin fiber observed in the nucleus by electron microscopy [1] a higher-order structure, formed by the coiling of the polynucleosome filament, has to be supposed. The need of the presence of proteins from the H1 family for the maintenance of the higher-order structure of chromatin is well documented [2–5] as well as the different effects of peptides from this protein [6, 7].

One of the candidates to produce changes in chromatin superstructure is the group of non-histone proteins. High-mobility-group (HMG) proteins are the best characterized of the non-histone protein fractions (for reviews, see [8, 9]). They can be classified into two groups, HMG-1 and 2 with a molecular mass of approximately 29 kDa and HMG-14 and 17 of approximately 10 kDa. Little evidence is available about the function of HMG-1 and 2, they are able to act upon the topology of DNA [10] and this property can be located [11] in the central domain of its polypeptide chain [12, 13]. For HMG-14 and 17 a function in the maintenance of the structure of active chromatin [14] has been proposed.

Previous studies have mainly been focused on the association of HMG proteins with monomer nucleosomes enriched in transcribed sequences after micrococcal nuclease digestion at low or intermediate ionic strength and fractionation according to their selective solubility in solutions containing either 0.1 M NaCl [15] or divalent cations [16–18]. Nevertheless the relation of the presence of HMG proteins to the stability of higher-order structure of chromatin has not been studied although it has been reported that HMG 14 and 17 do not disturb this structure when added to a solution of chromatin [19].

In the present paper advantage has been taken of the fact that, by extraction of chromatin at physiological ionic strength after mild digestion with micrococcal nuclease at low temperature, the chromatin fragments are stable in solution through protein–protein or protein–DNA interactions, despite having their DNA cleaved [20]. This property can be used to test conditions or chromatin components, such as acetylated histones, that may act upon the stability of higher-order structure of chromatin [21]. Using this approach the behaviour of HMG-containing chromatin has been analyzed in the present study. A method of preparing rat liver nuclei, allowing the extraction of soluble chromatin at physiological ionic strength and at 40 mM NaCl, has been devised. With this method the ionic conditions do not change during preparation and analysis of chromatin samples. As in the detection of HMG proteins in mononucleosomes [22], immunological methods have been employed in the present study in order to avoid the extraction of HMG and electrophoretic quantification.

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Abbreviations. HMG proteins, high-mobility-group proteins; $PhMeSO_2F$, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; ELISA, enzyme-linked immunoabsorbant assay.

Enzymes. Micrococcal nuclease (EC 3.1.31.1), ribonuclease A (EC 3.1.27.5), proteinase K (EC 3.4.21.14).

EXPERIMENTAL PROCEDURES

Rat liver nuclei and chromatin preparation

Two methods of nuclei preparation were used, differing in the concentration of salt present in the buffers. All operations were carried out at 4 °C unless otherwise stated. At 140 mM ionic strength rat livers, either fresh or frozen at −80 °C, were

homogenized in 0.34 M sucrose, 110 mM KCl, 30 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$), 10 mM Tris/HCl, pH 7.4 in a Potter-Elvejhem homogenizer. After filtering though a double layer of nylon gauze the suspension was layered on the top of a double sucrose cushion, consisting of equal volumes of 2.1 M sucrose, 110 mM KCl, 30 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $PhMeSO_2F$, 10 mM Tris/HCl, pH 7.4 in the lower part and 1.2 M sucrose, 110 mM KCl, 30 mM NaCl, 0.5 mM $PhMeSO_2F$, 10 mM Tris/HCl, pH 7.4 in the intermediate layer. The homogenate was centrifuged through the double cushion at 24000 rpm in a Beckman SW28 rotor. The nuclei were resuspended in a buffer having the same ionic composition of the lower cushion except that it had 0.34 M sucrose instead of 2.1 M. At lower ionic strength a similar method was used. In this case the homogenization buffer was composed of 0.34 M sucrose, 30 mM KCl, 10 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM EDTA, 0.5 mM $PhMeSO_2F$, 10 mM Tris/HCl, pH 7.4, the intermediate cushion buffer had the same composition except that the sucrose concentration was 1.2 M and had no EDTA and the lower cushion buffer had 2.1 M sucrose, 1 mM $CaCl_2$, no spermidine and spermine and the rest of components identical to the other two buffers. The yield of nuclei by both methods was similar (approximately 1 mg chromatin DNA/g fresh liver) and the state of the nuclei was checked by light microscope, appearing as a non-aggregate suspension of nuclei with a well-preserved membrane.

The nuclei were resuspended at an approximate concentration of 2 mg/ml in DNA and were immediately used for nuclease digestion. Micrococcal nuclease (Boehringer Mannheim, 15000 U/mg) digestion was carried out in 1.25 mM $CaCl_2$ and 4 °C. The digestion was stopped with the addition of EDTA to 2.5 mM and the nuclei were incubated with 50 μg/ml bovine pancreatic RNase A (Sigma) for 20 min at room temperature.

Chromatin was allowed to solubilize by dialyzing the digested nuclei against buffer A (0.2 mM EDTA, 0.5 mM $PhMeSO_2F$, 10 mM Tris/HCl, pH 7.4) with either 140 mM or 40 mM KCl. The suspension was centrifuged for 20 min at 2500 rpm in a bench centrifuge and the supernatant used for the different studies. The sedimentation of chromatin was carried out in 5–20% sucrose gradients in the corresponding buffer. 0.5-ml samples were loaded onto the gradients and centrifuged in a Beckman SW40 Ti rotor for either 90 min or 180 min at 39000 rpm at 4 °C. The gradients were monitored at 254 nm through a continuous-flow monitor (ISCO) and 1-ml fractions collected. Sedimentation coefficients were measured in relation with parallel gradients where maize endosperm polysomes were run.

DNA samples were analyzed in native conditions in vertical agarose gels (20 × 25 cm) using the Tris/borate/EDTA (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.0) buffer system and run at 150 V for 5 h. Gels in denaturing conditions were also run in agarose cast in 30 mM NaCl and run in 30 mM NaOH, after alkaline denaturation of the samples, at 100 V for 10 h. Gels were stained with ethidium bromide and photographed with a Polaroid MP-4 Land camera under 302-nm illustration. Protein electrophoresis was carried out in polyacrylamide slab gels using the buffer system of Laemmli [23].

Immunological methods

The proteins used in our study were HMG-1, HMG-2 and HMG-14 and 17. HMG-1 and 2 were prepared by extraction of calf thymus nuclei with 5% perchloric acid [24] and separation with ion-exchange chromatography in carboxymethyl-Sephadex C-25 (Pharmacia) according to Goodwin et al. [25]. HMG-14 and 17 were prepared as a joint fraction by extraction of calf thymus nuclei with 0.35 M NaCl, 0.5 mM $PhMeSO_2F$, 0.1 mM Tris/HCl, pH 7.0 [25] and purification by ion-exchange chromatography in carboxymethyl-Sephadex C-25 according to Goodwin et al. [26].

Antibodies were elicited in rabbits by injection of the pure protein following essentially the procedure of Bustin et al. [27] except that 0.5 mg protein was used in the injections. Antisera titer was determined by passive hemagglutination [28] and enzyme-linked immunoabsorbant assay (ELISA). Antibodies against each protein fraction were purified by immunoabsorption to proteins attached to CNBr-activated Sepharose (Pharmacia) [29] and eluted with 0.1 M glycine/HCl, pH 2.8.

Immunological detection methods used were essentially protein blotting, and immunodot. Protein blotting was carried out by transferring the proteins separated by electrophoresis to nitrocellulose filters (BA85, Schleicher and Schüll) using electrophoretic transfer in a Trans-Blot (Bio-Rad) device. Immunodot detection was carried out by using a Hybri-Dot (BRL) device. Chromatin fractions, adjusted at constant DNA concentration, were loaded directly on the paper. Dilutions of stock solutions of purified proteins were also loaded in separate wells. Filters were incubated with the corresponding antibodies at concentration between 5 μg/ml and 10 μg/ml, with ^{125}I-labelled protein A (New England Nuclear) and washed as described [30]. After autoradiography the films were developed and scanned with a Chromoscan 3 (Joyce-Loebl) microdensitometer.

RESULTS

Preparation of nuclei and chromatin

The presence of HMG proteins was studied in chromatin fractions prepared by micrococcal nuclease digestion in nuclei and fractionated by centrifugation in sucrose gradients. The conditions, physiological ionic strength and low temperature, were chosen in order to produce a minimal disturbance in the higher-order structure of chromatin. A method was designed that allows the preparation of nuclei at physiological ionic strength with a yield comparable to other available methods and the solubilization of chromatin at the same ionic strength. The method consists in homogenization of nuclei at physiological ionic strength in the presence of a chelating agent for divalent cations (EDTA) to avoid the action of endogenous nucleases. Centrifugation is carried out in only one step through a double sucrose cushion. In this way a yield of 1 mg DNA in nuclei/g rat liver was typically obtained. The nuclei appear intact, non-aggregated and free from cytoplasmic contaminants as seen by light microscope and without any detectable degradation of DNA or proteins.

In order to study the behaviour of chromatin fragmented and analyzed at a lower ionic strength (40 mM KCl) a strategy of nuclei preparation similar to that presented before was taken. In this case spermine and spermidine were added in the homogenization buffer but they were absent in the intermediate buffer and, instead, 1 mM $CaCl_2$ was present in the lower cushion. The yield of nuclei was similar to that of the previous method. However, in this case some chromatin was solubilized from the nuclei after incubation in the presence of $CaCl_2$ with no micrococcal nuclease added, indicating the presence of some residual endogenous nucleolytic activity.

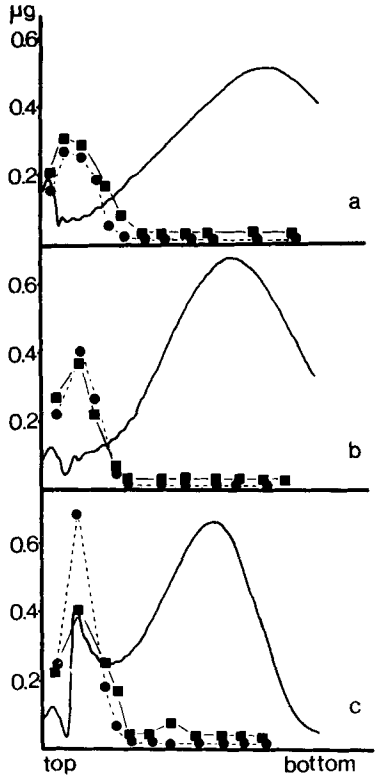

Fig. 1. *Time course of chromatin digestion by micrococcal nuclease at physiological ionic strength: distribution of HMG proteins.* Rat liver nuclei were digested at 4 °C with micrococcal nuclease. Aliquots were withdrawn at different times and the reaction was stopped by the addition of EDTA to 2.5 mM. The solubilized chromatin was sedimented through 5–20% sucrose linear gradients at 140 mM NaCl. Sedimentation was carried out during 90 min at 39000 rpm in an SW 40 Ti rotor. HMG proteins were detected by immunodot in fractionated chromatin obtained after 30 (a), 60 (b) and 180 (c) min of digestion with 170 units micrococcal nuclease/mg DNA at the same ionic strength. Detection of the proteins was carried out by using antibodies against HMG-1 and HMG-2 (■) and HMG-14 and 17 (●)

The chromatin was solubilized from nuclei after micrococcal nuclease digestion at 4 °C. Depending on the extent of digestion up to 80% of the total DNA was extracted as a chromatin suspension. The soluble chromatin was loaded onto 5–20% sucrose gradients containing the same buffer used for the extraction. A broad distribution is observed whose maximum *s* value decreases as the digestion time increases. At longer digestion times, and corresponding to the period of maximum chromatin solubilization, a bimodal pattern is observed that is conserved during a prolonged period of time (Fig. 1). The value of the fastest sedimenting material is stabilized at 80 S. This figure corresponds to approximately 15 nucleosomes. However, in fractions corresponding to high nucleosome multiplicities smaller oligomers are also present as can be seen by analyzing the DNA extracted from different gradient fractions (Fig. 2). The DNA fragments had an asymmetrical distribution with its maximum value around 12 nucleosomes. By running agarose gel electrophoresis in denaturing conditions it appears that a greater number of small oligonucleosomes are present in the denaturing gels, a consequence of the presence of a large number of nicks in the DNA [31]. It can be observed (result not shown) that the distribution of nucleosomal fragments is centered between 8 and 6 nucleosomes in the denaturing gel, and this value is conserved in the most digested samples chosen from the 80-S fractions. The distribution of histone H1 was measured in the different fractions. Fractions near to the top of the gradient lacked histone H1 and its proportion increased along the gradient reaching a plateau at the maximum of the 80-S material. The proportion of H1 in these fractions was never higher than 80% of the H1 present in total nuclei samples.

Immunodetection of HMG proteins

HMG proteins are present in a relatively low proportion in chromatin, they run in SDS gel electrophoresis very near to abundant protein fractions such as histone H1 for HMG-1 and histone H3 for HMG-17, making difficult their positive identification and quantitative determination. To solve this problem the presence of HMG proteins in different chromatin fractions was measured using immunological techniques. Antibodies were raised in rabbits using the method of Bustin et al. [27]. Three groups of proteins were injected: HMG-1, HMG-2 and HMG-14 and 17. The similarity of the HMG-14 and HMG-17 sequences and their possibly similar functions suggested that it was not necessary to try to separate these proteins. Similarly the cross-reaction between HMG-1 and 2 was very strong in most of the sera and they were used indistinguishably. The titer of the sera were measured by passive hemagglutination and ELISA and a specific IgG fraction was purified by immunoabsorption [29]. The antibodies were tested for cross-reaction with other chromatin proteins. In Fig. 3 the immunoblots of typical antibodies against calf thymus HMGs, against rat liver HMGs and against total chromatin proteins are shown. HMG-1 antibodies react in all the cases studied only against this protein and against HMG-2, the crossreaction in the opposite direction was also always observed. Antibodies against HMG-14 and 17 reacted only with these two proteins from calf thymus. When analyzing blots containing rat liver proteins a band corresponding to HMG-17 is clearly observed; however, no reaction appears in the HMG-14 zone but in a position corresponding to a protein having a slightly lower mobility. In fact not all HMG proteins from rat liver have been isolated and in total HMG extractions from rat liver HMG-14 is hardly seen when analyzed by gel electrophoresis [32, 33].

In order to quantify the presence of HMGs in the different chromatin fractions the immunodot technique was used. Samples of pure protein were also included in the filters in order to allow a quantification of the measurements. These values give the lower limit of protein content in the samples as it is possible that in chromatin the antigenic reaction is decreased. This method was also applied to measure the relation of HMG proteins present in the soluble and pellet fractions after nuclease digestion. This measurement was carried out by extracting both fractions with 5% perchloric acid and with 0.35 M NaCl. Both methods gave similar results and the relative proportion of HMG-1 between supernatant and pellet concentration was 0.93 ± 0.05; 1.09 ± 0.05 and 1.09 ± 0.05 for digestion times giving sedimentation patterns equivalent to gradients a, b and c in Fig. 1.

The result of sucrose gradient fractionation for three digestion times at 40 mM KCl is shown in Fig. 4 where the profile of absorbance at 254 nm is compared with the quantity of HMG present in the different fractions. It is possible to see that the proteins are concentrated in fractions of lower *s* values as compared with the bulk of chromatin. When the

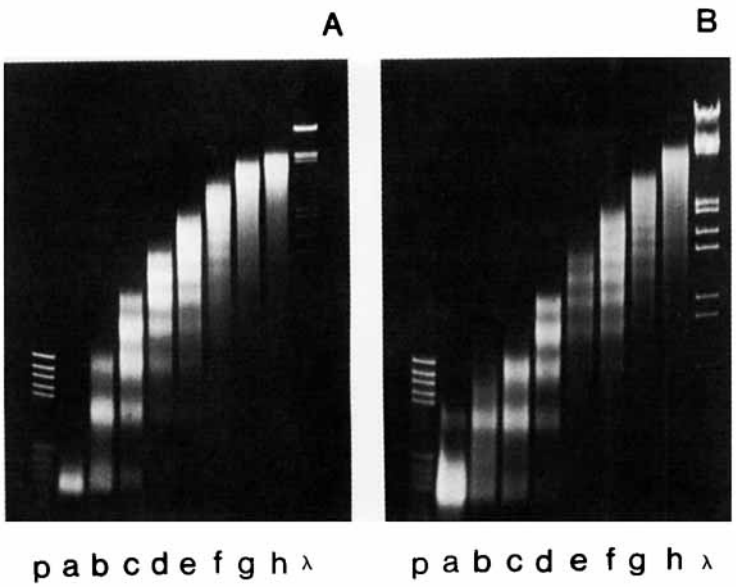

Fig. 2. *Agarose gel electrophoresis of DNA from different fractions of chromatin gradients at two digestion times.* (A) Samples corresponding to gradient (a of Fig. 1). (a–h) Fractions 3, 5, 7, 9, 11, 14, 17 and 19 from a total of 24 fractions. (B) Samples corresponding to gradient (c of Fig. 1). (a–h) fractions 3, 4, 7, 9, 11, 15 and 18. Sample p is plasmid pBR322 DNA digested with *Hae*III. Sample λ is phage λ DNA digested with *Hind*III and *Eco*RI

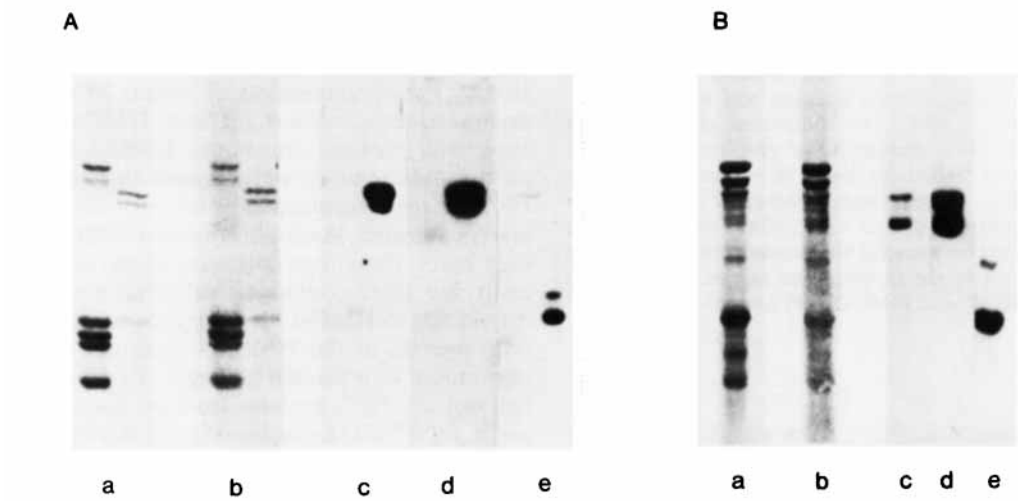

Fig. 3. *Immunological detection of proteins fractionated by polyacrylamide gel electrophoresis.* Proteins were transferred to nitrocellulose paper, incubated with the corresponding affinity-purified antibodies and detected with the ^{125}I-labelled protein A. (A) Controls of total rat liver histones and calf thymus HMG proteins. (B) Rat liver nuclei 0.35 M NaCl extract. (a) Gel stained with Coomassie blue; (b) blot stained with amido black; (c–e) blot incubated with antibodies against HMG-1, HMG-2 and HMG-14 and 17, respectively

digestion is allowed to proceed for longer times the material concentrates to smaller size products around the nucleosome monomer.

At 140 mM KCl the distribution of HMG proteins also differs from the bulk of the chromatin but in this case the material is concentrated in the slowly sedimenting material. The pattern of HMG distribution for the different gradient fractions is shown in Fig. 1 for three times of digestion. It is clear that most of the HMG-containing fractionated chromatin is located in the region of small oligonucleosomes.

In order to test whether the measured protein in the slowly sedimenting material is protein bound to nucleoprotein particles or free in solution, chromatin digested at 40 mM or 140 mM KCl was run in a sucrose gradient for 18 h in order to resolve the mononucleosome from smaller material. The result is shown in Fig. 5. The material reacting with the anti-HMG antibodies runs with the monomer peak with a small proportion of the HMG protein running in front of it. Another question that was also asked was whether the compact structure of the slowly sedimenting material was not masking the antigenic determinants of HMG proteins, preventing their reaction with the antibodies. To test this possibility four fractions from the top of the 80-S material at different digestion times were brought to 5 mM NaCl and extensively digested with micrococcal nuclease in these conditions. No difference was found between the reaction

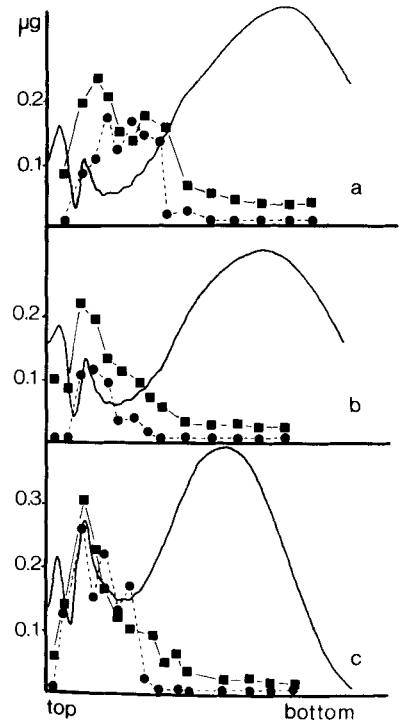

Fig. 4. *Distribution of HMG proteins in different chromatin fractions from a sucrose gradient sedimentation analysis at 40 mM KCl.* HMG proteins were detected by immunodot in fractionated chromatin obtained from rat liver nuclei after 30 (a), 60 (b) and 180 (c) min of digestion with 100 units micrococcal nuclease/mg DNA at the same ionic strength. Detection of the proteins was carried out by using antibodies against HMG-1 and HMG-2 (■) and HMG-14 and 17 (●)

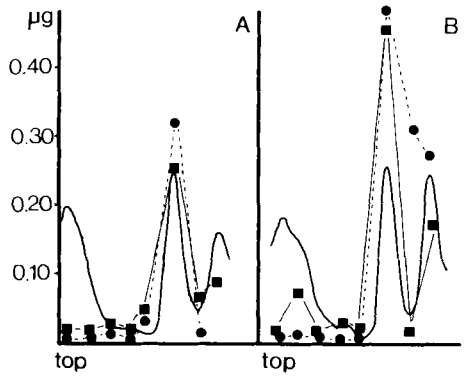

Fig. 5. *Distribution of HMG proteins in the slowly sedimenting chromatin fractions.* Samples equivalent to gradient (c) from Fig. 2 (A) and gradient (d') from Fig. 1 were sedimented for 18 h in order to resolve the chromatin material equivalent to nucleosome monomer or smaller. (■) HMG-1 and 2; (●) HMG-14 and 17

with antibodies at 140 mM NaCl, at 5 mM NaCl or at this salt concentration digested at the level of the mononucleosome.

DISCUSSION

The stability of chromatin containing HMG proteins has been studied in rat liver by sucrose gradient centrifugation after micrococcal nuclease digestion. A method has been devised in order to keep the global ionic strength unchanged during the experiment, to use preparative and analytical methods that perturb as little as possible the chromatin structure and to avoid the separation of fractions in function of their solubility in different buffers. The nuclei have been prepared at physiological ionic strength by a one-step centrifugation through a double sucrose cushion.

Several authors have used micrococcal nuclease digestion and partial solubilization of chromatin in order to study their enrichment in HMG proteins. The relation of these proteins with specific mononucleosome subfractions has also been studied [17, 34, 35]. In fact contradictory results have been obtained specially regarding the relation of HMG-14 and 17 with nucleosomes enriched in active gene sequences [18, 36, 37] and they have been explained in relation to their affinity to long core particles [38]. In our experiments the entire nuclear material is present during digestion and solubilization and care has been taken to avoid the exonucleolytic activity of micrococcal nuclease and changes in ionic strength that may favour unspecific interactions.

By using this method the distribution of the different HMG proteins in the chromatin fractions clearly departs from the distribution of the bulk of chromatin as measured by the absorbance at 254 nm. These proteins concentrate in the fractions corresponding to short oligonucleosomes or to the mononucleosome fraction when the digestion is more extensive. This effect is not due to the inaccessibility of the protein to the antibody in the larger fractions as when these are further digested at low ionic strength the reaction is not observed to increase. Two main hypotheses can be formulated to explain this phenomenon. It could be possible that all the solubilized HMG proteins exchange to the lower oligonucleosome material during the digestion and solubilization periods on that these proteins produce an increased instability of the chromatin superstructure leading to the disruption of the HMG-containing 80-S material. An overall rearrangement of HMGs does not occur in our conditions as we always find the same proportion of these proteins in the solubilized chromatin and in the pellet. The fact is that at physiological ionic strength the 80-S stable material contains a lower proportion of histone H1 than that found in nuclei and, therefore, it is possible to assume that there still exist sites for locating HMG proteins. It appears either that in our conditions the sites of attachment of HMG proteins are preserved during the digestion, and they produce an increased instability to the chromatin superstructure, or that during the process these proteins exchange to the fragments having a more open structure. The differences observed at the two ionic strengths studied can be also explained by these two mechanisms. In any case our experiments indicate that HMG proteins tend to accumulate in the most destructured regions of chromatin.

Similar behaviour is observed for HMG-1 and 2 and for HMG-14 and 17. The functions of these two groups of proteins may be different but both of them seem to affect the stability of higher-order structure of chromatin. It is then possible either that these proteins may help to fold the 30-nm chromatin fiber by producing local points of instability, which may be related to the effects on the DNA superhelicity in the case of HMG-1 and 2 [10, 11], or that by being located at precise chromatin zones, such as actively transcribed nucleosomes, as has been proposed [14], they produce a local destabilization of the chromatin superstructure.

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