The distribution of histone H1 subfractions in chromatin subunits

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ABSTRACT

Rat liver chromatin was digested with micrococcal nuclease to various extents and fractionated into nucleosomes, di and trimers of nucleosomes on an isokinetic sucrose gradient. In conditions under which degradation of linker DNA within the particles was limited, the electrophoretic analysis of the histone content showed that the overall content of H1 histone increased from nucleosomes to higher order oligomers. Moreover, the histone H1 subfractions were found unevenly distributed among the chromatin subunits, one of them, H1-3 showing most variation. A more regular distribution of these subfractions was found in subunits obtained from a more extended digestion level of chromatin. It is suggested that the H1 subfractions differ in the protection they confer upon DNA.

INTRODUCTION

The basic structure of chromatin is now well established. The fundamental subunit or nucleosome, consists of an histone octamer made of two each of H2A, H2B, H3, and H4, around which a fragment of DNA is wrapped (1-6). The DNA length varies from 140 to 200 base pairs in rat liver (7).

The very lysine rich histone H1 does not seem to be required to maintain the nucleosome core structure. Several reports (8-9-10-11-12) using various approaches lead to the conclusion that this histone interacts with the variable part of the DNA repeat called linker DNA ; its size varies from 0 to 60 base pairs in rat liver (7). Studies of the enzymatic degradation of DNA in H1 depleted and complete chromatin, suggest that this histone protects the linker DNA (9-10). In fact histone H1 is made of a central globular part and of two tails (13) which may have different binding properties to the nucleosomal DNA. Preliminary studies of the interaction of H1 with superhelical DNA (14) and chromatin subunits (15) would support this idea.

Regardless of its location in chromatin, a striking feature of histone H1 is its variability among tissues and species (16-17-18). Histone H1 is in fact a family of related proteins which may differ by several amino-

acid residues(19). A certain physiological role for histone H1 subfractions, could depend on the location of a given fraction on the chromatin fiber. Varshavsky et al (11) have studied the distribution of whole histone H1 on the chromatin subunits. They found that an heterogeneous population of nucleosomes was obtained from a micrococcal nuclease digest of chromatin : two discrete structural types of nucleosomes could be identified by electrophoresis and they differ by both their length of DNA and H1 content. Two more recent reports showed that nucleosomes could be distinguished by the H1 subfractions distribution (20-21).

The present study addresses the question of whether the various histone H1 subfractions confer different properties upon nucleosomes to which they are bound. We have studied the extent of H1 binding to nucleosomes and higher order oligomers obtained at low and high levels of micrococcal nuclease digestion. An uneven distribution of histone H1 subfractions is found among the chromatin oligomers. The variations observed for each H1 subfraction as a function of the digestion suggest a difference in the protection conferred upon linker DNA.

MATERIALS AND METHODS

Nucleosomes were prepared by micrococcal nuclease digestion either from nuclei or from non sheared chromatin prepared as described by Noll (22).

a) - Preparation of nuclei and chromatin

Rat liver nuclei were isolated by the method of Hewish and Burgoyne (1). PMSF^{*} was added to 0.5 mM in all extraction buffers to inhibit proteolytic activity (23).

Chromatin was prepared from nuclei by a brief digestion with micrococcal nuclease (Boehringer, 30 units/10⁸ nuclei for 30 sec. at 37°C). Digestion was carried out in 0.34 M sucrose in buffer A plus 1 mM CaCl₂ and stopped with 2 mM EDTA and chilling on ice. Nuclei were then lysed in 0.2 mM EDTA pH 7, 0.1 mM PMSF. Large fragments of chromatin obtained were further purified through a sucrose cushion in 1 mM TEA^{***} pH 7.4, 0.1 mM PMSF at 82,000 g for 15 hours.

b) - Micrococcal nuclease digestion of nuclei and purified chromatin

150 units of micrococcal nuclease were added to 10⁸ nuclei suspended in digestion buffer. Chromatin solution in 1 mM TEA pH 7.4, 0.1 mM PMSF

^{*}PMSF : Phenylmethane sulfonyl fluoride

^{***}TEA : Triethanolamine

was digested by nuclease (1 unit/A₂₅₈ unit) in the presence of 0.1 mM CaCl₂. Incubation took place for the given amount of time at 37°C and was stopped by adding EDTA to 1 mM. The kinetics of digestion were followed by TCA precipitation; the undigested material was allowed to precipitate on ice for 10 min. before pelleting by centrifugation at 10,000 g for 10 min. The acid-soluble material was measured by its absorbance at 258 nm and corrected for hyperchromicity. The percent of digestion was plotted versus time (fig. 1).

c) - Sucrose gradient sedimentation

Samples of digested material (nuclei or purified chromatin) were layered on an isokinetic sucrose gradient as described by M. Noll (3) (V_m = 33 ml, V_t = 36 ml, 5 % - 40 % sucrose). Sucrose solutions were made either in 1 mM phosphate buffer pH 7.4 or in 1 mM TEA pH 7.4, 1 mM EDTA, 0.1 mM PMSF for digested nuclei and chromatin respectively. The subunits were fractionated by centrifugation at 96,000 g for 20 hours in a SW 27 rotor. Gradients were analyzed by spectrophotometry and central fractions only were collected for each of the oligomers peaks investigated.

d) - DNA analysis

DNA was extracted from aliquots of the oligomer fractions. Samples were treated with RNase and proteinase K; DNA was obtained by phenol/chloroform extraction and ethanol precipitation according to the procedure of Bellard et al. (24). Electrophoresis of DNA samples was performed on 2 % agarose slab gels in running buffer (40 mM Tris-HCl pH 7.8, 20 mM sodium acetate, 1 mM EDTA). The gel were run at 50 mA for 3 hours at room temperature and stained in 0.5 µg/ml of ethidium bromide. Hae III restriction fragments of ØX 174 DNA (replicative form) (New England Biolabs) were used as size markers.

e) - Proteins analysis

The pooled fractions were dialyzed overnight against 0.1 mM PMSF and then lyophilized. The dried samples were dissolved in 62.5 mM Tris-HCl and pH 6.8, 2 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol and 0.001 % bromophenol blue as a marker (25). The proteins were denatured by immersing the samples for 1 to 2 min. in boiling water. SDS polyacrylamide slab gel electrophoresis was performed according to Laemmli (25). The slab gel consisted of a stacking gel (2 cm of length, 3 % acrylamide 12.5 mM Tris-HCl pH 6.8, 0.1 % SDS) and a separating gel (12 cm in length, 15 % acrylamide, 375 mM Tris HCl pH 8.8, 0.1 % SDS). The stacking gel and the separating gel were prepared from a stock solution of 30 % by weight of acrylamide and 0.8 % by weight of N, N'-bismethylene acrylamide. The electrode buffer pH 8.3 contained 25 mM Tris and 192 mM glycine, 0.1 % SDS. Electrophoresis were performed

at 35 mA for six hours. After fixation in acetic acid, methanol, H_2O (1 : 5 : 5 v/v) for 1 hour, slabs were stained with Coomassie blue R 250 0.1 % in the same solution for 1 hour and destained in 3 changes of 500 ml of acetic acid, methanol, H_2O (1 : 6 : 8 v/v) with constant agitation.

The concentration of bound dye was determined optically by scanning the slabs. The amount of protein in a given electrophoretic band was estimated by measuring the peak area, occasionally decomposed into gaussian peaks when overlapping occurred between two bands. For each sample the amount of histone H1 (total H1 and H1 subfractions) was expressed relative to histone H4 assuming that the inner histone content is constant. Staining intensity was calibrated by known quantities of histone fractions.

RESULTS

a) - Subunit fractionation

Subunits of chromatin were obtained either from nuclei or from chromatin purified as described above.

The time course of early nuclease digestion expressed as the amount of acid-soluble DNA released is shown on fig. 1. Arrows correspond to the two typical extents of digestion used in this study. Fig. 2 shows the sedimentation profiles obtained from these digests ; at low extent of digestion most of the

FIG. 1 - Purified chromatin digestion by micrococcal nuclease. (arrows correspond to 5 % and 16 % digestion).

FIG. 2 - Isokinetic sucrose gradient analysis of micrococcal nuclease digested chromatin.

material is found in the lower part of the gradient but as the digestion proceeds an increased amount of higher order oligomers and nucleosomes are produced. The fractions corresponding to monomers, dimers and higher order oligomers were collected.

b) - DNA analysis

The purity of the collected fractions was determined by agarose gel electrophoresis of their DNA content (fig. 3). It was found to be 95 % or more for monomer, 80-90 % for dimer, 60-70 % for trimer (which was mainly contaminated by tetramer).

The DNA length of purified chromatin was mostly in the range of 15 to 20 nucleosomes, only traces of fragments, lower in size than 10 nucleosomes but always higher than 5 could be detected on the gels.

The DNA lengths of nucleosomes obtained at 4 %, 6 % and 16 % acid-soluble DNA were 193, 183 and 183 base pairs respectively.

c) - Protein analysis

. Analysis of purified chromatin subunits

The SDS polyacrylamide gel patterns of the histones from chromatin and the different fractions studied are shown on fig. 4 and fig. 5.

FIG. 3 - 2 % agarose slab gel electrophoresis ; analysis of 5 % rat chromatin digested by micrococcal nuclease and of oligonucleosomes fractionated on an isokinetic sucrose gradient : slots 1,7, Hae III restriction fragments of ϕ X 174 DNA (replicative form) ; slot 2, rat liver purified chromatin ; slot 3, purified chromatin digest ; slots 4,5,6, monomer, dimer, trimer and tetramer.

The purified chromatin pattern shows all five histones. The core histones are clearly fractionated into four bands. Three electrophoretic subfractions of histone H1 are resolved as described by others (26-27-28). For convenience we have called these subfractions H1-1 H1-2 and H1-3 according to their relative electrophoretic mobilities (H1-1 being the slowest band). To rule out the possibility that these subfractions are degradation products due to proteolytic activity, we have incubated purified chromatin for 15 hours at 37°C.

FIG. 4 - SDS-polyacrylamide slab gel electrophoresis ; proteins analysis of oligonucleosomes obtained from a 5 % digested chromatin :
 (A) monomer, (B) dimer, (C) trimer, tetramer (D) > pentamer,
 (E) purified chromatin.

After this treatment the relative proportions of these three bands were unchanged.

None of these bands was released from chromatin by extensive washing in 0.35M NaCl but all of them were extracted in 0.6 M NaCl. In addition these three bands were found in a 5 % perchloric acid extract. All these data strongly suggest that these bands correspond to the H1 histone family.

The amounts of total H1 and H1 subfractions relative to H4 in mono, di, and trimer of nucleosomes were determined as described in Methods. As we can see in table I the amount of total histone H1 relative to H4 increases with the length of nucleosomes chain obtained from a 5 % digest of purified chromatin. A similar result has been reported by several authors analyzing the proteins associated with nucleosomes obtained by micrococcal nuclease di-

FIG. 5 - Scans of SDS-polyacrylamide slab gels ; (A) monomer, (B) dimer, (C) trimer-tetramer.

gestion (8-12-32-33) but the real meaning of this observation is still open to discussion. A more interesting observation concerns the amounts of the different subfractions of histone H1 in oligomers. Each of the three subfractions shows a significant increase from monomer to trimer. And the maximum value is found in each case for purified chromatin. However the variation of the relative amount depends on the nature of the subfractions. The most marked increase is observed for the subfraction H1-3 ; a 4 fold increase from monomer to purified chromatin is observed for this subfraction while a 2 fold increase is observed for the other fractions. Two hypothesis can be considered to explain the particular behaviour of H1 subfractions. Both of them involve an heterogeneous distribution of the various H1 subfractions along the chromatin fiber.

- 1 - Histone H1 has been found to be released from nucleosomes by extensive digestion of chromatin (9) (10) (11). This is due to degradation of the linker DNA to which the histone is thought to be bound. In our experimental conditions, the DNA of the monomer is found to be 190 base pairs long for a 5 % digest, suggesting that linker degradation is minimized. However this value represents the mean value of a monomer population which is obviously heterogeneous in size as seen from the broadness of the band in the electrophoretic pattern. This heterogeneity has been shown to originate from the linker (7). One cannot exclude however that within this population some linker degradation may have occurred during the digestion process, which would mainly affect the monomer particle. The results found for the distribution of H1 subfractions would be explained by assuming that the rate of the degradation of the linker depends on the particular H1 subfraction bound to it ; the linker bound to H1-3 would be preferentially degraded.

- 2 - On the other hand, histone H1 has been found to protect DNA against micrococcal nuclease digestion (9) (10) (11). A 8 fold increase of the rate of digestion of the DNA is observed on H1 depleted chromatin as compared to native chromatin (9). Again this process preferentially affects the DNA of the linker. In our experiment one can assume that there is a gradation in the protective effect of the various H1 subfractions. H1-3 would be the most efficient in this process. The result would be a preferential production of nucleosomes lacking this fraction at early time of digestion.

The above alternative can be resolved by analyzing the subfractions distribution in nucleosomes obtained from a higher level of digestion. In the first hypothesis, further digestion would accentuate the process and gives rise to monomers fully depleted of H1-3 ; in the second hypothesis increasing the digestion would finally result in the attack of the most resistant regions, and in the production of an increased number of monomers with H1-3. As a consequence a more homogeneous distribution of this subfraction would be observed among the fractionated samples. Table I (right side) strongly supports the second hypothesis. Indeed the histone composition of fractions obtained from a 16 % digest of purified chromatin shows that histone H1-3 is then evenly distributed among mono, di and trimers and the extent of the variation of the other subfractions is reduced as compared to the 5 % digest. It can be noticed however that subfraction H1-2 is the less affected by this process.

These results indicate that the various histone H1 subfractions have a different protective effect against micrococcal nuclease digestion.

	Digest			Digest			Chromatin
	5 %	5 %	5 %	16 %	16 %	16 %	
	Monomer	Dimer	Trimer	Monomer	Dimer	Trimer	
H1(tot.)	0.38±0.02	0.51±0.01	0.66±0.02	0.59±0.02	0.67±0.02	0.70±0.02	0.77±0.02
H1_1	0.27±0.03	0.37±0.01	0.46±0.01	0.41±0.04	0.48±0.05	0.50±0.05	0.50±0.03
H1_2	0.08±0.02	0.09±0.01	0.11±0.01	0.09±0.01	0.10±0.01	0.11±0.01	0.16±0.02
H1_3	0.030±0.005	0.050±0.001	0.09±0.02	0.09±0.01	0.09±0.01	0.09±0.01	0.11±0.02

TABLE I - H1 and H1 subfractions amount relative to H4 in mono, di and trimers obtained from a 5 % digest (left side) of chromatin.

Histone H1-3 seems to be the most efficient in this process followed by H1-1 and then H1-2.

. Analysis of subunits fractionated from intact nuclei

In the experiments described above we have used purified chromatin prepared according to Noll et al. (29).

If the properties found for histone subfractions are specific to their mode of interaction with chromatin, one should expect similar properties when intact nuclei are digested with micrococcal nuclease.

Table II displays the relative amounts of histone H1 subfractions in particles obtained from a 5 % and 15 % digest of nuclei.

A behavior similar to that described in the preceding paragraph is observed

	Digest			Digest			Nuclei
	5 %	5 %	5 %	15 %	15 %	15 %	
	Monomer	Dimer	Trimer	Monomer	Dimer	Trimer	
H1(tot)	0.33	0.52	0.62	0.35	0.63	0.67	0.76
H1_1	0.19	0.30	0.35	0.20	0.38	0.38	0.43
H1_2	0.11	0.14	0.17	0.09	0.18	0.20	0.22
H1_3	0.03	0.08	0.10	0.06	0.07	0.09	0.11

TABLE II - H1 and H1 subfractions amount relative to H4 in mono, di and trimers obtained from a 5 % digest (right side) and from a 15 % digest of nuclei.

although the variation between 5 % and 15 % is less marked. The relative amounts of H1 subfractions increase from monomer to higher order oligomer, H1-3 showing the most extended variation. The extent of these variations is lowered upon further digestion, leading to similar conclusions as before.

DISCUSSION

The results described above lead to the following comments. First, as previously described in other reports (9-12-21-29-30), we find that the distribution of whole histone H1 among nucleosomes and oligomers of nucleosomes is uneven. A selective loss of histone H1 as the digestion proceeds has been proposed as a mechanism to explain this result (9) (11). The level of digestion (% of solubilized DNA) is an important parameter in such studies. In the above reports, "stripped" nucleosomes were always obtained from a high level of digestion (26 % in Noll et al. (9), 19 % in Gaubatz et al. (12)).

In our experiments we observe an irregular distribution of histone H1 in nucleosomes and their oligomers at the earliest time of digestion when 5 % of the DNA is rendered acid soluble. At this low digestion level, the degradation of the linker, or the part of it which carries histone H1, cannot account for the lower amount of this histone in nucleosomes as compared to higher order oligomers. Moreover, when the digestion level is increased to 15 % acid-soluble DNA, a more even distribution of H1 is observed. This result can be best explained if one assumes that histone H1 is unevenly distributed along the chromatin fiber, and that, at very early stage of digestion, the mechanism of the production of nucleosome is predominantly based on the protective effect of histone H1 against nuclease attack. In other words, since less than one copy of histone H1 per nucleosome is present in nuclei (21), the first nucleosomes to be produced by a mild digestion will be those lacking histone H1. Further digestion will gradually increase the proportion of nucleosomes with histone H1, resulting in a progressive increase of the mean relative amount of this protein. Then the final step of linker degradation already described will take place producing nucleosomes lacking H1 but with a shorter DNA length.

More striking is the distribution of H1 subfractions in nucleosomes and higher order oligomers. SDS polyacrylamide gels reveal three subfractions of H1 (H1-1, H1-2, H1-3) in rat liver chromatin. The amounts of these subfractions is again a function of the size of the subunits, and the extent of variation differs for each subfraction. The greatest variation is observed for H1-3. According to the preceding mechanism these variations

can be attributed to a differential protective effect of the various H1 sub-fractions. The fraction H1-3 offers most protective effect against micrococcal nuclease digestion, followed by H1-1 and H1-2. Although studying a different system and by another approach, Albright et al. (21) were led to similar conclusions concerning a differential protective role of two H1 sub-fractions.

Very recently, the sensitivity of trout testis chromatin regions to micrococcal nuclease has been shown to be related to proteins associated with them : the HMG-T- containing linker regions were more susceptible to the nuclease attack than H1 containing linkers (31).

Further studies will be necessary to elucidate the mechanism by which this phenomenon occurs and to know if it is related to physiological properties.

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