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EFFECT OF HISTONE COMPOSITION ON THE STABILITY OF CHROMATIN STRUCTURE

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The mode of fragmentation of chromatin by micrococcal nuclease has been studied in nuclei from different sources at physiological ionic strength and low temperature. During digestion, the size of chromatin was reduced until an average S value of 95–100 (hen erythrocyte) or 60–65 (rat liver) was attained. The accumulation of these structures correlated with the period of maximum solubility (80%), indicating that the bulk of chromatin behaved in this manner. Further digestion did not result in a corresponding decrease in S value but in a bimodal sedimentation pattern. As opposed to this behavior, chromatin containing actively acetylated core histones showed a continuous variation in size during the digestion. Indirect immunoprecipitation of chromatin by anti-H5 antibody and sheep anti-rabbit antibody revealed that the acetylated chromatin is partially depleted of H5.

Introduction

Among the factors known to be involved in the maintenance of the higher order structure of chromatin, the role played by the H1-like histones has probably been the best studied. Previous work from this laboratory indicated that the size of soluble erythrocyte chromatin of an average length of 24 nucleosomes is relatively resistant to digestion by micrococcal nuclease at physiological ionic strength, even though its DNA is cleaved [1]. The maintenance of this structure was found to be dependent on the stoichiometry and integrity of histones H1,H5. In this report we present evidence suggesting that this behavior is a property of the bulk of chromatin in situ. However, the average length of chromatin displaying such a behavior is shorter in H1- than in H1,H5-containing chromatin. On the other hand, chromatin fibers containing actively acetylated core histones show a continuous shift to smaller sizes during the digestion, behaving like extended or H1,H5-depleted chromatin.

Materials and Methods

Cells

Red blood cells from phenylhydrazine-treated and non-treated hens were obtained and purified as described [2]. Immature erythrocytes were incubated with 200 μCi/ml of sodium [^{3}H]acetate (New England Nuclear; 500 mCi/mmol) for 1 h at 37°C [3]. After chilling the suspension, sodium *n*-butyrate [4] was added to a concentration of 2 mM and the cells were washed three times with NKM [2] containing 2 mM sodium *n*-butyrate. The specific activity of the labeled material relative to

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

the DNA concentration was found to be 500 dpm/μg DNA.

Nuclei and chromatin

Hen erythrocyte nuclei were prepared as described previously [1] in buffer A (110 mM KCl/30 mM NaCl/0.2 mM $MgCl_2$/10 mM triethanolamine hydrochloride/0.4 mM PMSF, pH 7.4), and 2 mM *n*-butyrate was included in the buffers used for the labeled cells. Nuclei were resuspended at a DNA concentration of around 5 mg/ml and digested with micrococcal nuclease (Worthington) (60 units per mg DNA) in the presence of 1 mM $CaCl_2$. After digestion EDTA was added to 2.5 mM and nuclei were dialyzed overnight with rotatory agitation against 140 mM or 40 mM KCl in buffer B (0.2 mM EDTA/0.4 mM PMSF/10 mM triethanolamine hydrochloride, pH 7.4). Soluble chromatin was recovered from the supernatant of a 10 min centrifugation at 3000 rev./min and the pellet was re-extracted once more by the same procedure. Acid-soluble DNA was measured as described [5].

Rat liver nuclei were isolated essentially as described [6] except that 110 mM KCl/30 mM NaCl/1 mM PMSF/0.2 mM EDTA/10 mM triethanolamine hydrochloride, pH 7.4, was used throughout. Nuclease digestion and chromatin extraction was carried out as described for hen erythrocyte nuclei with the exception that the samples were treated with 50 μg/ml RNAase A (Sigma; heated at 80°C for 10 min).

Samples of chromatin, 250–500 μl (approx. 1.0 mg/ml), were centrifuged through 5–20% linear sucrose gradients at the indicated salt concentration in buffer B in a Beckman SW40 Ti rotor at 39000 rev./min. Gradients were monitored at 254 nm. Apparent sedimentation coefficients were calculated as described [1].

Antibodies

The preparation of rabbit anti-H5 and sheep anti-rabbit antibodies, and their purification by affinity chromatography, has been described [7]. The antibodies had no detectable nuclease or protease activity, and the anti-H5 antibodies did not cross-react with H1 [7]. Chromatin fractions were reacted with anti-H5 IgG (1.75 mg/mg chromatin DNA) in buffer B containing 2 mM sodium *n*-butyrate, 0.1% Nonidet P40, 0.1 mg/ml bovine serum albumin (Pentex, Miles) and the corresponding sucrose concentration for 1 h at room temperature followed by overnight incubation at 4°C. A 5-fold excess sheep anti-rabbit antibody to anti-H5 IgG was then added and it was incubated for 3 h at 4°C. After centrifugation the radioactivity in the immunosupernatant and in the immunoprecipitate was determined. Around 95% of the radioactivity in the supernatant was precipitable by 18% (w/v) trichloroacetic acid. The percentage of chromatin in each fraction was determined after gel electrophoresis by scanning the core histone bands with a Vitatron microdensitometer equipped with a Spectra-Physics integrator [8].

Modified histones were resolved by electrophoresis in 15% polyacrylamide slab gels containing 8 M urea and 0.45% Triton X-100 [9]. The stacking gel was 7.5% polyacrylamide and contained 0.1 mg/ml of protamine. Aliquots of chromatin were treated with 5% trichloroacetic acid to extract histones H1 and H5 and were dissolved in 0.125 M HCl/5 M urea/2 M acetic acid/5 μg/μl protamine, and run at 10 V/cm for 18 h. 15–19% SDS-polyacrylamide gradient slab gels were made as described [8]. Fluorography [10] was carried out by exposing presensitized [11] XR-5 Kodak films to (New England Nuclear) Enhance-treated gels.

Results

Digestion of hen erythrocyte nuclei

The solubilization of chromatin from hen erythrocyte nuclei was studied at physiological ionic strength and low temperature and the results are summarized in Fig. 1. The percentage of chromatin extracted from nuclei increased with the time of digestion until a maximum of about 80% was reached, and decreased thereafter (Fig. 1B). The drop in solubility was not observed in parallel aliquots if the extraction was carried out at 40 mM KCl. Since chromatin is partially unfolded at the lower ionic strength [1], these results suggested that the insolubility observed could reflect the properties of the higher-order structure of chromatin.

To characterize this phenomenon further, we determined the size of the chromatin extracted at 140 mM KCl as a function of the extent of diges-

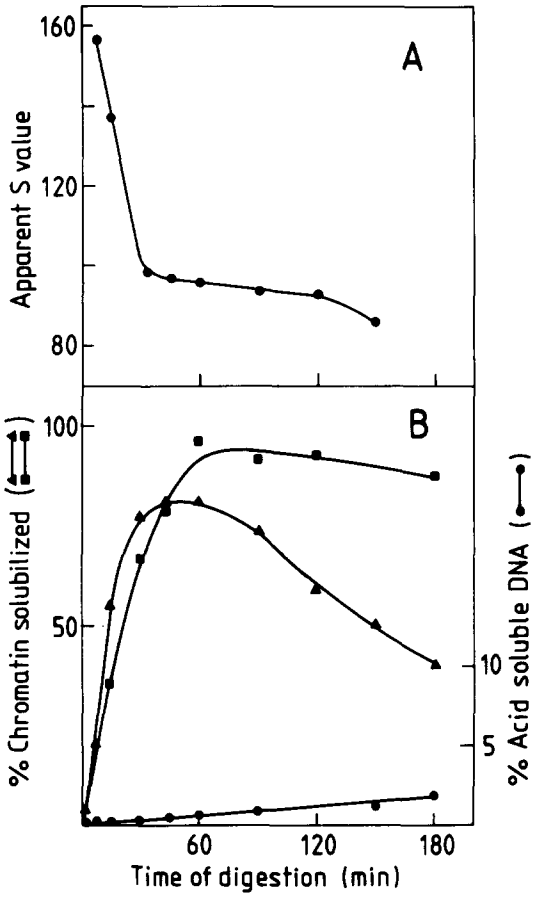

Fig. 1. Solubilization of chromatin after a time-course digestion of hen erythrocyte nuclei by micrococcal nuclease. (B) Chromatin was extracted either at 140 mM (▲——▲) or 40 mM KCl (■——■) in buffer B. The amount of acid-soluble DNA (●——●) is also shown. (A) S-value of the fast sedimenting chromatin material during the course of digestion.

tion. Fig. 1A shows that the S value of chromatin decreased with the increase in solubility, suggesting a random action of the nuclease. At the maximum of solubility the size of the main product reached an average S value of 95–100 which did not significantly change with time (Fig. 1A); and the distribution of DNA fragments in the soluble and insoluble fractions was indistinguishable (average length of 24 nucleosomes). Continued digestion resulted in a decrease in solubility and in the relative amount of the 95–100 S chromatin while the proportion of slowly sedimenting material increased. Chromatin under this lower S-value peak represented a mixture of small oligonucleosomes, mostly monomers, which were depleted of histones H1 and H5 (Table II, see also Ref. 1). Eventually, depleted mononucleosomes constituted the only material solubilized under these conditions. This pattern of sedimentation was not observed when chromatin was extracted at 40 mM KCl. In this case the distribution of fragments shifted continuously towards lower S values during equivalent time of digestion (not shown). The constancy in size of the faster sedimenting chromatin was not due to inactivation of the nuclease, since DNA extracted from the 95–100 S material was shown to be progressively cleaved.

Because the behavior of chromatin digested in situ was very similar to that of chromatin digested in solution [1], we determined whether our results were due to the fact that chromatin was spontaneously solubilized, and its digestion occurred in solution rather than in nuclei. Nuclei were digested for 1 h and quickly centrifuged; the amount of solubilized material was determined after the addition of EDTA to the supernatant. Only 12% of chromatin was found to be spontaneously solubilized (as compared to 80% after longer extraction times—12 to 15 h—in the presence of EDTA), and most of the material consisted of small oligonucleosomes. Therefore we conclude that our results reflect the properties of chromatin digested in situ, and that the bulk of chromatin behaves in this fashion.

Digestion of rat liver nuclei

The nucleated erythrocyte is a rather unique system because it contains histone H5 [12,13] and its chromatin is highly condensed. We considered that the behavior of this chromatin could perhaps be also unique and not be representative of a general property of the higher-order structure of chromatin. Therefore, we extended our observations to rat liver nuclei and performed types of study similar to those described above for the erythrocyte nuclei. Briefly, the degree of solubility and the decrease in size of chromatin as a function of the extent of digestion was found to be similar to that of the erythrocyte. However, the average size of the chromatin fragments refractory to the nuclease attack was found to be smaller, sedimenting with an average S value of 60, corresponding

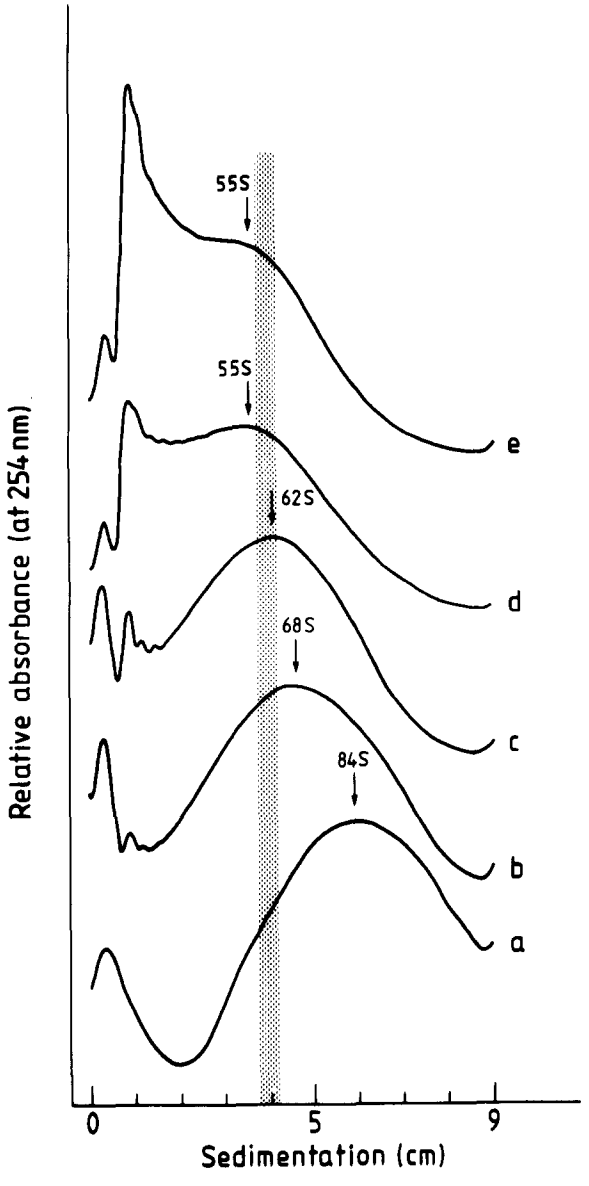

Fig. 2. Characterization of chromatin extracted from rat liver nuclei during the course of digestion by micrococcal nuclease at 140 mM KCl. Sucrose gradients containing 140 mM KCl were run for 130 min. a–e: 20, 60, 90, 120 and 150 min of digestion. The shadowed area corresponds to the S-value of a fragment of 15 nucleosomes.

to an average DNA length of 14–15 nucleosomes (Fig. 2). In addition, the larger chromatin fragments were not as stable against nuclease digestion as were those from erythrocyte chromatin. The peak near the top of the gradients consisted of non-dialyzable ribonucleoprotein particles resulting from the RNAase digestion of chromatin (see Materials and Methods).

Behavior of chromatin containing acetylated histones

The structural properties of chromatin manifested by our experimental approach reflect only the behavior of the bulk of chromatin. For this reason we have also used radioactive tracers to characterize regions of chromatin of special interest. Since histone acetylation and chromatin activity are related in time and in space [3,14], it was relevant to find out whether chromatin containing actively acetylated histones might have an altered structure. For these experiments red blood cells from anemic animals were pulse-labeled with

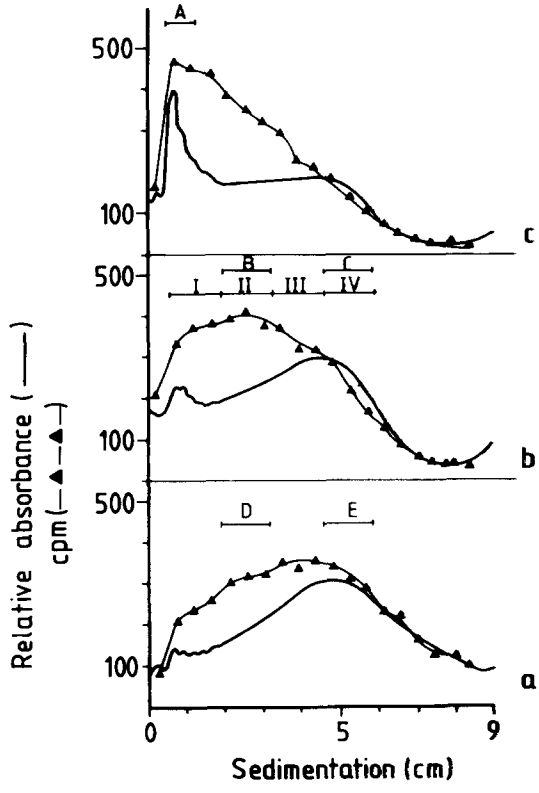

Fig. 3. Sedimentation profiles of chromatin solubilized at 140 mM KCl after micrococcal nuclease digestion of immature erythrocyte nuclei pulse-labeled with [^{3}H]acetate. Chromatin extracted in 140 mM KCl in buffer B was sedimented in 140 mM KCl containing sucrose gradients run for 80 min. 2 mM sodium *n*-butyrate was included in all buffers. a–c: 35, 60 and 90 min of digestion, respectively. The horizontal bars represent the fractions analyzed in Fig. 4 and in Tables I and II.

TABLE I

RELATIVE [^{3}H]ACETATE CONTENT IN HISTONES H4 AND H2B FROM CHROMATIN FRACTIONATED BY SIZE

Fractions I–IV correspond to the pools indicated in Fig. 3b. Values presented are percentages of label incorporated by the mono-, di-, tri-, and tetraacetylated species (Ac1–Ac4) and were determined by densitometry of fluorographed urea-Triton X-100 gels (Fig. 4A). Only the areas of the first four bands were used in the calculation. The rest of the bands, especially in the H2B region, were not clearly identified.

Histone subfraction	Fraction			
	I	II	III	IV
H4 Ac1	54.7	56.3	66.8	76.7
2	24.3	22.0	18.5	17.1
3	11.0	11.8	7.9	4.0
4	10.1	9.9	6.7	2.2
H2B Ac1	34.6	42.5	49.7	60.4
2	24.2	25.6	28.7	18.9
3	22.9	20.3	18.8	15.1
4	18.2	11.6	2.8	5.5

[^{3}H]acetate for 1 h. To inhibit histone deacetylation [15–17], *n*-butyrate was included during nuclei isolation and in all subsequent steps. The vast majority of the radioactivity was incorporated by the core histones (Fig. 4C) at the lysine side-chains and no detectable label was incorporated as *N*-acetylserine in H4 (Fig. 4A, B). Therefore, the ^{3}H radioactivity can be used to follow the behavior of chromatin undergoing active post-synthetic acetylation [3]. Fig. 3 shows the sedimentation properties of chromatin extracted at 140 mM KCl during the course of digestion by micrococcal nuclease. Clearly, the radioactive chromatin sedimented more slowly than the bulk of the material, which behaved much in the same fashion as chromatin from mature cells. In addition, the size of the actively acetylated chromatin decreased continuously, with no indication of a pause similar to the 95–100 S.

The broadness of the radioactivity profiles suggests that chromatin containing histones modified to different extents may be digested at different rates. This notion is supported by the results shown in Fig. 4, where the content and extent of acetylation of the histones from chromatin fractionated by sucrose gradients (Fig. 3b, pools I–IV) is compared. The data are also expressed quantitatively for H4 and H2B in Table I. There is a clear tendency for chromatin containing higher proportion of hyperacetylated histones to be digested at a

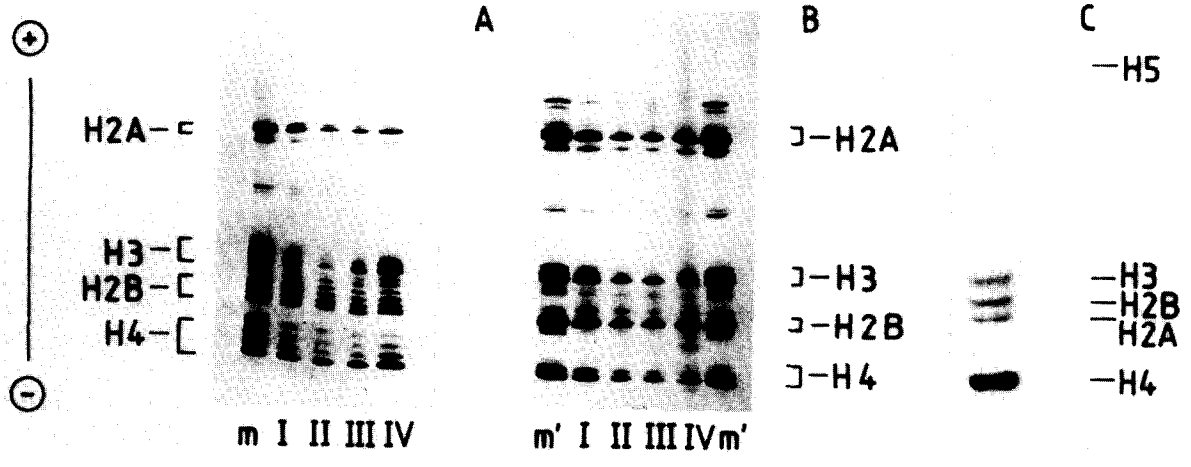

Fig. 4. Degree of histone acetylation in chromatin fractionated by size. Immature erythrocytes were labeled for 1 h with [^{3}H]acetate and chromatin was fractionated as indicated in Fig. 3. A and B are, respectively, fluorography and Coomassie-blue stained urea-Triton X-100 gels of the 5% trichloroacetic acid-treated chromatin fractions indicated in Fig. 3. The brackets show the bands analyzed; other minor components have not been identified. m is a sample of chromatin from immature erythrocytes labeled with [^{3}H]acetate in presence of 5 mM *n*-butyrate; m', core histones from mature erythrocytes. C. Fluorography of the chromatin proteins separated by electrophoresis in SDS-polyacrylamide gels. Note that radioactivity is associated only with the core histones.

higher rate. This is also supported by results from experiments similar to those described here, in which the cells were pulsed with [^{3}H]acetate in the presence of *n*-butyrate. In this case a higher proportion of the label was found in the hypermodified forms of the histones (Fig. 4A, m) and the peak of radioactivity was more symmetric (not shown). A higher digestion rate of chromatin containing hyperacetylated histones from cells treated with butyrate has been previously shown [18]. Interestingly, the turnover of acetyl groups of the hypermodified H4 molecules was much higher than that of the H2B, although the relative proportion of modified H2B was lower (Fig. 4A, B). This can be seen by comparison of the relative uptake of [^{3}H]acetate during 1 h by each of the H4 and H2B subspecies in the absence (Fig. 4A, I–IV) or presence (Fig. 4A, m) of *n*-butyrate. The higher proportion of [^{3}H]acetate uptake in the monoacetylated H4 (H4 Ac1) is likely to reflect the slower acetate turnover, since the cells were pulsed for a rather long time [19]. Part of the acetylation in H4 Ac1 appears to be constitutive [20] and perhaps unrelated to the functional acetylation, since its relative proportion is enriched in the bulk of chromatin (Table I).

Since H1 and H5 are involved in the higher-order structure of chromatin, we determined whether the lower degree of stability of acetylated chromatin could also reflect changes in the content of H5,H1. This was examined by indirect immunoprecipitation of chromatin fragments of different length (Fig. 3 A–E) with affinity chromatography-purified rabbit anti-H5 and sheep anti-rabbit antibodies. Table II shows that, whereas the major part of chromatin in each fraction (with the exception of the smaller fragments which are depleted of H1 and H5 (Table II, Ref. 1) did precipitate, the amount of radioactivity precipitated was in no case higher than 60% and decreased with the size of the fragments. The radioactivity remaining in the immunosupernatant could be quantitatively precipitated with trichloroacetic acid, indicating that it was protein-bound. In other words, the amount of chromatin precipitable with the antibodies decreased with the increase in the extent of histone acetylation. Since the antigenic determinants are distributed along the H5 molecule Ref. 21 and Torres, S. and Ruiz-Carrillo, A., unpublished data), it is unlikely that possible modifications of H5, occurring concomitantly to core histone acetylation, may be responsible for a decreased affinity of the antibody used. These results strongly suggest that actively acetylated chromatin is, at least partly, depleted of H5.

TABLE II

INDIRECT IMMUNOPRECIPITATION OF CHROMATIN FRACTIONS BY ANTI-H5 ANTIBODIES

Fractions A–E correspond to the pools indicated in Fig. 3. Ratio was calculated by densitometry of histones from the corresponding chromatin fractions after electrophoresis in SDS-polyacrylamide gels. The amount of chromatin precipitated was determined by comparing the area under the core histone peaks from aliquots of the immunoprecipitate and immunosupernatant chromatin after electrophoresis in SDS-polyacrylamide gels.

Chromatin fraction	H1+H5/ core histones	[^{3}H]Acetate radioactivity precipitated (%)	Chromatin precipitated (%)
A	0.03	8.4	12.0
B	0.14	31.4	99.6
C	0.27	59.5 (8.0) [a]	95.6
D	n.d.	33.6	87.2
E	n.d.	54.2	91.8

[a] Amount of radioactivity precipitated when neutral rabbit IgG instead of anti-H5 IgG was used in the immunoprecipitation.

Discussion

We have shown that the sedimentation properties of chromatin digested in situ by micrococcal nuclease strongly depend on the ionic conditions used for the extraction. Thus, while the extraction of chromatin at physiological salt concentration reveals the production of a major product (although with a broad size distribution) which accumulates in time, solubilization at lower salt molarity (40 mM KCl) precludes the detection of such a structure. The similar behavior observed in situ and in solution [1] indicates that no gross differences in conformation exist between these states. The strength and the range of interaction among the chromatin fragments that make up these structures is certainly modulated by the type of H1-like

histone present. Hence, not only the length of the equivalent polynucleosome chain is smaller in rat liver (H1-containing), but also its stability against digestion appears to be lower than in the erythrocyte (H1,H5-containing). The differential stability may be related to the fact that H1 has a lower affinity for chromatin than H5, and while H1 appears to exchange between chromatin fragments [22], H5 does not [23].

Our results cannot distinguish whether the stability of the chromatin fiber is not constant along its length but changes more or less periodically, or whether a minimum length of the polynucleosome chain is necessary for the maintenance of a more stable structure. Neither of these two possibilities requires a priori that the chromatin fiber should fold in a discontinuous manner as has been proposed on the basis of electron micrographs of chromatin subjected to low salt concentrations and possibly stretched during spreading [24,25]. However, these electron microscope observations and the result of some cross-linking experiments [26] could be best reconciled with the first notion of chromatin organization.

Although the relative accessibility of acetylated chromatin to the nuclease may be higher, this cannot be the sole cause for its different behavior. A higher accessibility of chromatin should result in a similar pattern of fragmentation as seen for the bulk of chromatin albeit at relatively shorter times of digestion and this was not observed. Instead, our results suggest that acetylated chromatin also has a reduced stability. The importance of the H1-like histones in the process of chromatin condensation is further emphasized by the finding that actively acetylated chromatin has a reduced content of H5. Since it has been found that nucleosomes containing H1 or H5 are closely interspersed in the erythrocyte chromatin (Torres, S. and Ruiz-Carrillo, A., unpublished data), we think that it is not likely that the lack of H5 is compensated for by an increased content of H1. We cannot rule out, however, that the reduced stability of the acetylated chromatin could be due both to the presence of H1 and the modification of the core histones. In any event, these changes lead to a more open structure; this also appears to be true for the transcriptionally active regions of chromatin [27]. It will be interesting to find out whether this acetylated chromatin is enriched in actively transcribing sequences.

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