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Influence of Distamycin, Chromomycin, and UV-irradiation on Extraction of Histone H1 from Rat Liver Nuclei by Polyglutamic Acid

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Abstract—Rat liver nucleus histone H1 was fractionated by polyglutamic acid (PG) in the presence of distamycin A (DM) or chromomycin A_3 (CM). In the absence of the antibiotics, PG extracts from the nuclei about half of the nuclear H1. DM or CM added to the nuclei in saturating concentrations weakens the binding potential of most of H1. Titration of nuclei with DM shows that the number of binding sites for DM in the nuclei is less than in isolated DNA by only 20-25%, and this difference disappears after treatment of nuclei with PG. The lower CD value of DM complexes with nuclei compared to that of DM complexes with free DNA is evidence of a change in the DM–DNA binding mode in nuclear chromatin. About 25% of total histone H1 is sensitive only to DM and ~5% is sensitive only to CM. Half of the DM-sensitive H1 fraction seems to have a different binding mode in condensed compared relaxed chromatin. A small part of H1 (~3%) remains tightly bound to the nuclear chromatin independent of the presence of the antibiotics. Subfraction H1A is more DM-sensitive and H1B is more CM-sensitive. UV irradiation of nuclei results in dose-dependent cross-linking of up to 50% of total H1, which is neither acid-extractable nor recovered during SDS electrophoresis. PG with DM extracts only about 3% of H1 from UV-stabilized chromatin. DM treatment of the nuclei before UV irradiation results in extraction of the whole DM-sensitive H1 fraction (~25%), which in this case is not stabilized in the nucleus. A hypothesis on possible roles of the found H1 fractions in chromatin structural organization is discussed.

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Key words: cell nucleus, chromatin, histone H1, polyglutamic acid, distamycin, chromomycin, UV irradiation

Histone H1 is one of the main chromatin proteins localized in the nuclei of all eukaryotes. It plays a key role in the chromatin structure and function, but many of its functions are not yet quite clear. Its primary function is participation in the internucleosomal interactions providing chromatin compaction [1]. However, there is an opinion that H1 only stabilizes the higher structures [2].

The interaction between H1 and DNA is mainly ionic and is divided between the three of its domains: the *N*- and *C*-termini and the globular part. However, in specific cases, e.g. in active chromatin, it may be bound to DNA by the *N*- and *C*-termini only [3]. Histone H1 has at least two sites for binding of the globular domain on the nucleosome [4]. It has also been shown that two H1 molecules can possibly bind to one nucleosome [5].

Histone H1 is represented by several protein subtypes. The subtypes have different primary structures of the *N*- and *C*-termini and possess a relatively conservative globular region. The quantitative ratio of subtypes varies in the course of differentiation, embryonal development, during spermiogenesis, under hormone-induced changes in gene expression, etc. [6]. It is also known that the subtypes of histone H1 apparently have different abilities for chromatin compaction [7, 8].

A new concept of the role of H1 in chromatin structure and function has been formed in connection with the intensive study of the dynamic behavior of H1 in living cells at different stages of ontogenesis [9]. The observed protein mobility reflecting the strength of its

Abbreviations: CM, chromomycin A_3; DM, distamycin A; PG, polyglutamic acid; PMSF, phenylmethylsulfonyl fluoride; TEA, triethanolamine.

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binding to chromatin significantly varies depending on the structure of the *C*-terminus, posttranslational modifications, and competition with other nuclear components for the binding site on chromatin. A change in the strength of H1 binding is one of the earliest events leading directly to changes in chromatin structure and activity [10].

The strength of H1 binding in chromatin is also influenced by the nucleotide composition of DNA, interaction with other proteins, and modification of DNA and core histones. In particular, it has been shown that H1 is preferably bound to AT-rich DNA regions [11]. Division of the H1 pool into fractions based on the strength of binding has been reported in [12, 13]. It is likely that the strength of binding of the H1 molecule also depends on its position in the supranucleosomal structure of chromatin. However, judging by the literature there is still almost no data on H1 fractions corresponding to some chromatin structures.

Previously it was reported that polyglutamic acid (PG), being a H1-specific dissociating agent, removes from the nucleus only some part of the H1 [14] and thus can be used for division of nuclear H1 into fractions with different binding strengths.

The goal of the present work was to fractionate histone H1 using PG by the strength of its binding to chromatin in interphase nuclei of rat liver with preserved supranucleosomal structure of chromatin, to study the influence on this fractionation of antibiotics distamycin A (DM) and chromomycin A_3 (CM) modulating the interaction between histone H1 and the AT- or GC-sites of DNA in chromatin, and to study the effect of stabilization of chromatin structure by UV irradiation on H1 extractability from nuclei.

MATERIALS AND METHODS

Isolation of hepatocyte nuclei. Experiments were carried out using female white rats weighing ~150 g. All operations were performed at 5°C. The rat liver was homogenized in a glass homogenizer with a Teflon piston in buffer A (15 mM TEA-HCl, pH 7.6, 80 mM KCl, 2 mM EDTA, pH 7.0, 0.2 mM spermine, 0.5 mM spermidine) containing 8% sucrose. For inhibition of protease activity, phenylmethylsulfonyl fluoride (PMSF) was added to the solution (0.2 mM final concentration) immediately before homogenization. The homogenate was centrifuged at 1500*g* for 10 min. Pellet was supplemented with concentrated sucrose solution (2.5 M) in buffer A (final sucrose concentration 2.1 M) and PMSF, followed by centrifugation at 50,000*g* for 45 min. The pellet of nuclei was washed with solution A and again sedimented at 1000*g* for 10 min. DNA and RNA concentrations were measured by spectrophotometry in an aliquot of nuclear hydrolysate with 5% $HClO_4$ [15].

Treatment of nuclei and H1 extraction. The nuclei were suspended in buffer A/4 (3.75 mM TEA-HCl, pH 7.4, 20 mM KCl, 0.5 mM K-EDTA, 0.05 mM spermine, 0.125 mM spermidine) to 330 μg/ml in DNA (1 mM nucleotides). During single extraction, DM (Sigma, USA; 5 mg/ml in ethanol) or CM (Sigma; 5 mg/ml in ethanol) and an equal volume of PG solution (Sigma; molecular weight 15-50 kDa) in buffer A/4 were added in different orders to the nuclear aliquots at concentrations creating different ratios of these substances with the nuclear DNA. The aliquots were incubated with each reagent for 15-30 min at 5°C. The addition of $MgCl_2$ to CM-containing samples to 1.5 mM did not result in structural rearrangements in the chromatin within the nuclei (not illustrated). After incubation, NaCl was added to the reaction mixture (0.12 M final concentration) and centrifuged for 10 min at 10,000*g*. Centrifugation under these ionic conditions ($I \sim 0.14$) sediments the H1-containing chromatin. The pellet was washed again with PG solution of the same concentration containing 0.14 M NaCl, and the supernatants were combined. Extracted proteins were precipitated from the supernatant by adding trichloroacetic acid (TCA) to the final concentration of 20%, kept at −10°C for approximately 2 h, and collected by centrifugation for 10 min at 10,000*g*, then twice washed with cold acetone and air-dried. Histone H1 from the precipitates was extracted three times with 0.75 M $HClO_4$ followed by H1 precipitation with 3.5 volumes of acetone containing 0.03 volume of concentrated HCl overnight at −20°C, and then centrifuged for 10 min at 10,000*g*. The precipitates were washed with acetone, dried, and dissolved in electrophoresis buffer.

During step-wise extraction, the nuclei in buffer A/4 (330 μg/ml DNA) were incubated with DM (DM/DNA molar ratio = 0.01) and/or exposed to UV irradiation. Then equal PG volume was added (PG/DNA weight ratio = 6), incubated after each exposure for 15 min in the cold, and centrifuged for 10 min at 10,000*g*. The nuclear pellet was washed twice with PG of the same concentration and centrifuged in the same mode. The supernatants were combined (S1). The pellet was suspended in solution containing DM and PG in the same ratios to DNA, incubated for 15 min, and centrifuged. The pellet was washed twice with PG of the same concentration and centrifuged as well. The supernatants were combined (S2). The proteins from the supernatants and pellet were prepared for electrophoresis as described.

UV irradiation. The suspension of nuclei was exposed to UV irradiation for 30 min under a BUF-15 mercury quartz lamp with maximum emission of ~254 nm and power of 1.8 J/m^2 in a 5-mm layer under continuous stirring by a magnetic stirrer and cooling in ice.

SDS electrophoresis. Electrophoresis was performed in 15% polyacrylamide gel according to Laemmli [16]. Protein in the bands was assayed by the results of quantitative computer processing of electrophoregrams (Gel-

Pro Analyzer 3.1) using a calibration curve plotted for total H1.

Electron microscopy. Samples were fixed with 1% glutaraldehyde (neutralized to pH 7.0 by NaOH) at 4°C for 1.5 h and then with 1% OsO_4 for 1 h, dehydrated, and enclosed in Epon by the standard method. Ultrathin sections were obtained in an LKB III ultratome, contrasted with lead citrate, and viewed in a Hitachi 11B electron microscope at accelerating voltage of 75 kV.

Circular dichroism (CD) spectra. The spectra were measured in the region 250-360 nm using a modified Mark Y dichrograph (Jobin-Yvon, France) adapted for measurement of CD-absorbance of biological objects. Measurements were performed in a quartz cell with a 1-cm optical pathlength in the scanning mode. The accumulation time at one point was 2 sec; scanning pitch of the spectrum was 1 nm. The spectra were smoothed over five points using Origin software (MicroCal, USA). Concentrated DM solution was added in increasing amounts to the suspension of nuclei (35 μg/ml DNA) or the DNA isolated from them (at the same concentration) in buffer A and incubated for 15 min at 25°C, then the CD spectrum of the formed complex was recorded.

RESULTS

General characteristics of cell nuclei. Nuclei were isolated from rat hepatocytes in the presence of spermine and spermidine in EDTA buffer to maintain high molecular mass of DNA in the nuclei.

An electron micrograph of isolated nuclei (Fig. 1a) shows that chromatin is condensed and located mainly on the periphery of the nucleus and around the nucleolus. The fibrils and globules are 100-200 nm in diameter. They often form ring structures. When the nuclei are transferred to a buffer with lower ionic strength (A/4), chromatin is partially unfolded and occupies the greatest part of the nucleus. At the same time, chromatin fibrils 30-50 nm in diameter curled into numerous ring structures 100-150 nm in diameter (Fig. 1b). One can see that the 30-50-nm fibril sometimes also consists of rings. The observed rings

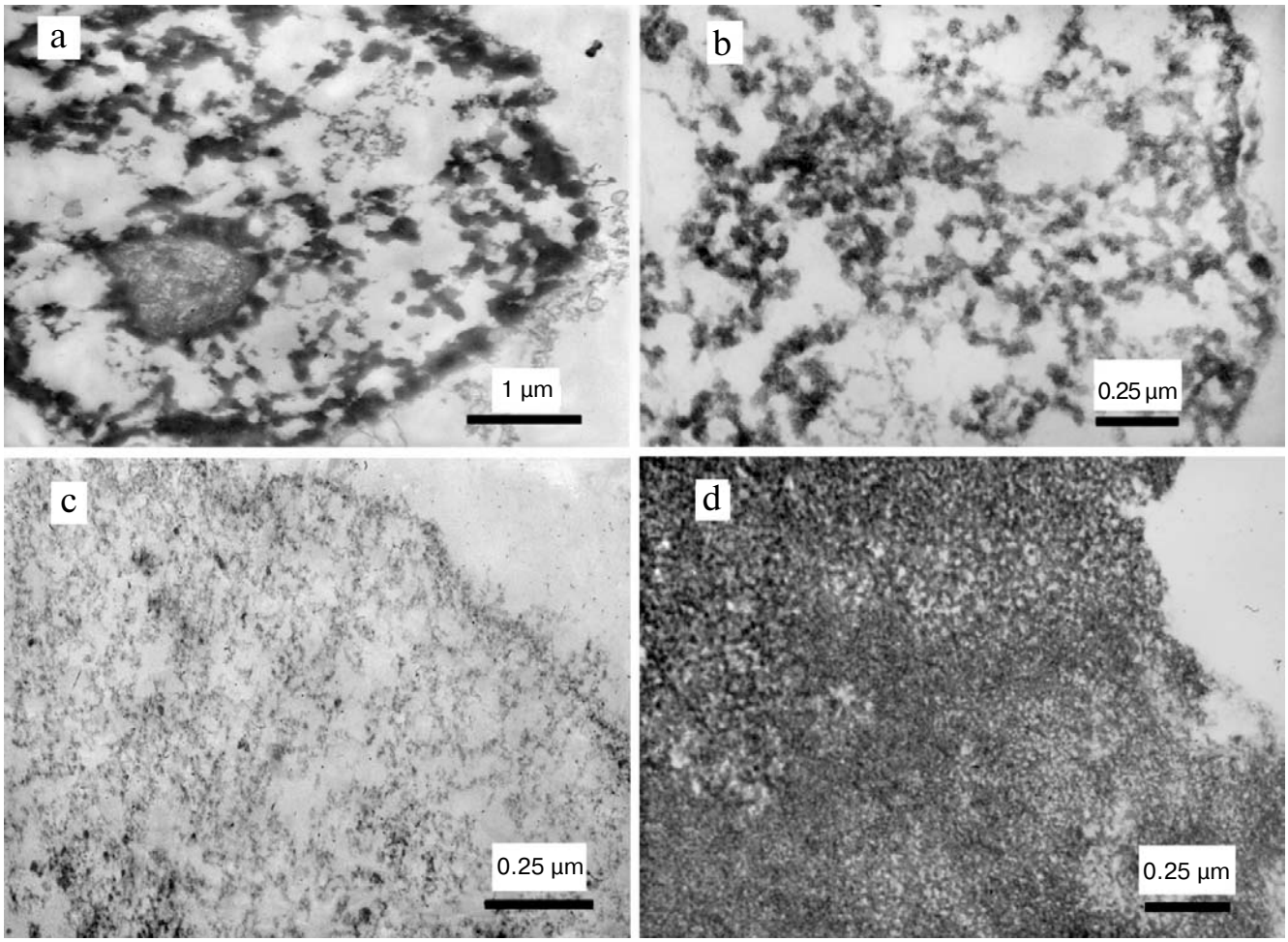

Fig. 1. Electron micrographs of isolated rat liver nuclei in buffers A (a) and A/4 (b) and PG-treated nuclei at PG/DNA = 6 (c) and 30 (d).

seem to be related to yet unknown internal organization of supranucleosome chromatin. We think that the chromatin structure in isolated nuclei in buffer A/4 corresponds mainly to the 30-nm level of organization.

The weight ratio of H1 isolated from the nuclei by $HC1O_4$ extraction to the nuclear DNA is ~0.2, corresponding to approximately one H1 molecule per nucleosome. Electrophoretic analysis of the extract showed the typical pattern of rat H1 consisting of three zones: H1A, H1B, and H1°.

Thus, the results of electron microscopy and biochemical studies suggest that isolated nuclei preserve H1 localization and binding mode typical of chromatin *in situ*.

Histone H1 extraction from cell nuclei by PG. It is known that PG, being a polyanion, interacts *in vitro* with the basic chromatin proteins. At the same time, PG first extracts H1 from chromatin and dissociates the positively charged ends of core histones [17].

Figure 2 (PG) shows the dependence of H1 extraction during the incubation of rat liver nuclei with PG at PG/DNA weight ratios of 0.24 to 60. The figure shows that the dependence is nonlinear, demonstrating heterogeneity of the initial H1 pool by its ability to be retained in the nucleus. At PG/DNA ≥ 30 : 1, the curve is close to saturation. The yield of H1 is ~50% of its total content in the nucleus, which is in agreement with results obtained previously for isolated metaphase chromosomes of HeLa cells [14]. Thus, PG allows to divide H1 of the interphase rat nucleus into weakly and strongly bound histones in approximately equal proportions.

We studied the effect of pH on H1 extraction from the nucleus at PG/DNA = 6. PG was inefficient at pH < 5.5. At pH 6.5-8.5, the yield of H1 changes little and rises only on further increase in pH. Thus, a certain spread of pH values around neutral one does not affect the yield of H1.

Electron microscopy of nuclei at weight ratio of PG/DNA = 6 shows (Fig. 1c) that the nuclear chromatin is decondensed to a certain intermediate state between supranucleosome and completely unfolded nucleosome structures. However, the 30-nm fibril swells but retains some elements of its packing: numerous ring, half ring, or loop structures. Increase in the ratio to PG/DNA = 30 does not result in further unfolding of the fibrils, and the entire structure appears to be pressed, probably due to dehydration of chromatin in the presence of high polymer concentrations (Fig. 1d).

DM/DNA interaction in interphase nuclei. The antibiotic DM interacts with DNA by incorporation into the minor groove mainly within AT-regions [18]. The interaction between DM and DNA directly in the interphase nuclei is practically unstudied. Therefore, we investigated in more detail the interaction between DM and chromatin in the interphase and PG-treated nuclei.

DM added to the rat liver interphase nuclei increases CD-absorbance at λ = 340 nm, demonstrating formation of a DM complex with nuclear DNA. Figure 3 shows the dependences of CD absorbance variation (ΔD) at λ = 340 nm on DM concentration for intact nuclei, PG-treated nuclei (PG/DNA = 6), and isolated DNA. It can be seen that the curves come to saturation at approximately

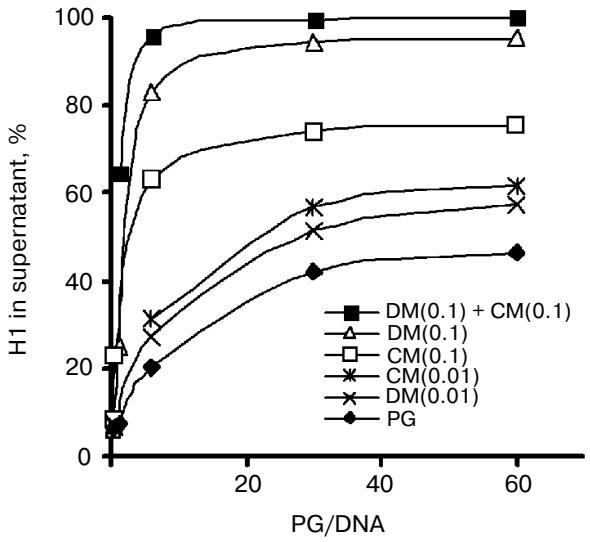

Fig. 2. Effects of DM and CM on extraction of histone H1 by polyglutamic acid. Mean values of parameters determined in 3-4 experiments differing less than 5% are presented. X-axis, PG/DNA weight ratio. Numbers in brackets are the antibiotic/DNA molar ratio (mole/mole nucleotides).

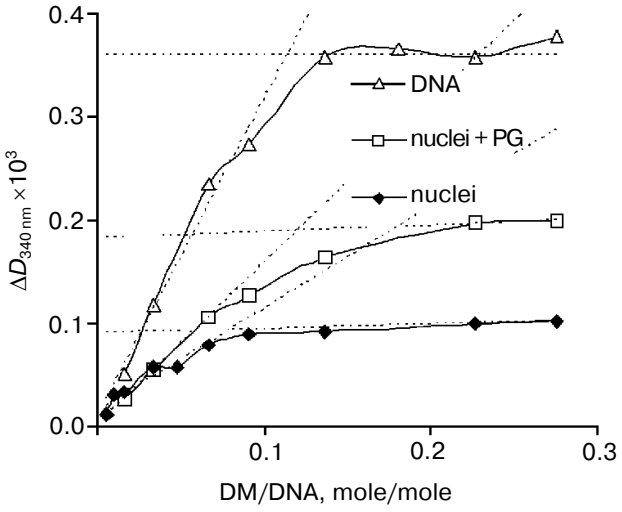

Fig. 3. Dependence of change in circular dichroism (ΔD at 340 nm) of DM–DNA complex in isolated rat liver nuclei, PG-treated nuclei, and isolated DNA on DM concentration. Measurements were performed at constant DNA concentration. Dotted lines show calculation of the size of the DM binding site.

equal DM concentrations. For free DNA and PG-treated nuclei, saturation is reached at DM/DNA (mole/mole nucleotides) = 0.11-0.12; for DNA in the intact nuclei, this ratio is ~0.08. This value corresponds to ~1 DM molecule per 4-5 base pairs in free DNA and in PG-treated nuclei, which is in agreement with the literature data on the size of the DM binding site [19], and per ~6 base pairs of DNA in the intact nuclei. Thus, the number of DM binding sites in the intact nuclei is about 25% less than in free DNA, according to the analogous value for isolated chromatin [20]. These findings suggest that DM molecules can be bound to the DNA of nuclei in the presence of proteins. Analogous data have been obtained for the interaction between chromatin and Hoechst dye, which is also bound to AT sites through the minor groove of DNA [21].

Figure 3 shows that the maximum CD values at 340 nm are significantly different at approximately equal saturations of DM preparations and the same DNA concentration. To account for these results, we suppose that the presence of proteins in the nuclear chromatin does not interdict DM binding but influences the type of complexes formed. It is probable that in the presence of proteins DM can be bound to DNA sites through only some of its amide groups. Indeed, the studies [22, 23] of the effects of DM analogs or modifications on formation of DNA complexes show that decrease in the number of amide groups results in formation of complexes with lower coefficient of extinction. This is similar to the situation during the interaction between DM and chromatin in the nucleus.

Effects of DM and CM on extraction of histone H1 from nuclei. Previous experiments *in vitro* have shown that DM competes with H1 for interaction with AT-sites of DNA in the SAR/MAR-sequences [13]. We studied the influence of DM on H1 extraction from intact nuclei. Our experiments show that DM *per se* does not extract H1 from chromatin, because histone H1 is not found among supernatant proteins after centrifugation of the nuclei pre-incubated with DM (not illustrated). The electron-microscopic analysis showed no changes in the chromatin structure of DM-treated nuclei compared to the control (not illustrated). However, incubation of nuclei with DM in saturating concentration (DM/DNA = 0.1) results in a considerable increase in H1 extraction into solution during their treatment with PG (Fig. 2, curve DM (0.1)). At PG/DNA < 6, the yield of H1 in the presence of DM increased almost linearly, demonstrating the lower heterogeneity of interaction between most H1 and DNA in the nucleus. So, at PG/DNA = 6 the amount of H1 in the extract was already 85% of its total content in the nucleus, i.e. almost 4-fold higher than its yield under the action of PG alone. During further increase in the PG/DNA ratio to 60, the amount of extracted H1 increased more slowly and reached a plateau in the region of 95%. The remaining 5% of histone H1, not depending on the action of DM, was extracted from the nuclear pellet by treatment with $HClO_4$. Note that electron-microscopic analysis in the presence of DM revealed no changes in the structure of PG-treated nuclear chromatin.

It follows from these results that DM destabilizes H1 interaction in the interphase nucleus not only in the AT-rich SAR/MAR-regions but also in much of the DNA sequences not enriched in AT-pairs. Indeed, it was reported that DM could interact with all DNA sites irrespective of their nucleotide composition, though with quite different binding constants [18, 24]. It follows from Fig. 2 that DM also affects the strength of H1 binding in the sites, from which it can be removed by increasing polyanion concentrations.

We have found that the antibiotic CM specific to GC sequences and, like DM, bound to the minor groove of DNA [25] also significantly enhances H1 extraction when added to the nuclei at the ratio of CM/DNA = 0.1 (Fig. 2, curve CM (0.1)). However, the maximum level of extraction at PG/DNA = 60 is only 75%, i.e. much lower than for DM. On one hand, this suggests that CM contributes to H1 extraction not only from GC-rich but also weakly GC-enriched sequences. On the other hand, since the minor groove in AT-sequences is narrower than in GC, it may be sterically inaccessible for CM, or the interaction with it may have a very low association constant [26]. Therefore, it may be supposed that the 25% amount of H1 not extracted in the presence of CM is located on the nucleosome linkers containing only AT-rich sequences. On the whole, the results are in agreement with the literature data on enrichment of linker sequences with AT-pairs [27, 28].

Comparison of the DM (0.1) and CM (0.1) curves (Fig. 2) shows that they are of similar character and in both cases the extracted H1 can be represented as two fractions: the main fraction, easily extracted by low PG concentration (PG/DNA < 6), and the minor fraction, which in both cases is ~10% of nuclear histone H1 and is extracted at PG/DNA > 6. The latter is less sensitive to antibiotics or, probably, more tightly bound in chromatin for a reason not depending on the nucleotide composition.

It was also shown that the action of CM and DM at once resulted in complete dissociation of H1 (Fig. 2, DM (0.1) + CM (0.1)). Consequently, 5% of H1 not extracted in the presence of DM (Fig. 2, DM (0.1)) can be removed in the presence of CM and seems to be located in the linkers carrying GC-rich DNA sequences. Indeed, it has been reported that some of the H1 subtypes are preferably bound to the GC-rich DNA regions [29, 30]. At the same time, a small portion of H1 (~3% of the total content) under these conditions is extracted only at PG/DNA > 6, which is evidence of the greater strength of binding of this H1 fraction to chromatin irrespective of nucleotide composition.

Analysis of the findings leads to a conclusion that: the action of DM and CM in chromatin of the interphase

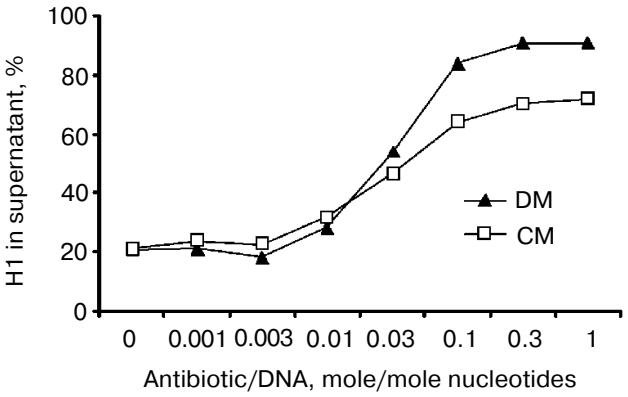

Fig. 4. Dependence of histone H1 extraction from nuclei by polyglutamic acid (PG/DNA = 6) on DM and CM concentrations. The mean values of parameters determined in three experiments differing less than 5% are presented.

nucleus is spread to the large H1 pools; these pools are overlapped to a considerable degree; in the presence of CM, PG extracts from the nucleus 20% less H1 than in the presence of DM, irrespective of PG concentration (PG/DNA = 6-60); among 50% of H1 more tightly bound in the nucleus (not extracted by PG only), ~5 and 25% are exceptionally sensitive to CM and DM, respectively. Thus, in spite of the known preference of H1 binding to the AT-rich DNA sequences, our data demonstrate the existence of an H1 fraction (~5%) tightly bound to the GC-rich linkers. The other 20% removed at maximum PG concentrations can also be divided, approximately in halves, into fractions more sensitive to DM or to CM. Some of the H1 (among these 20%) may be tightly bound in the nucleus irrespective of the nucleotide composition of the DNA.

Figure 4 presents curves of histone H1 extraction during DM or CM titration of nuclei followed by PG treatment (PG/DNA = 6). One can see that the curves have similar profiles, and active displacement of histone H1 with the involvement of antibiotics in both cases begins only at their molar ratio of DNA >0.003, corresponding to approximately one antibiotic molecule per nucleosome. Probably, below this ratio the antibiotics are incorporated into places on chromatin where histone H1 is either absent or bound particularly tightly irrespective of the nucleotide composition of DNA. Further increase in antibiotic concentrations to the antibiotic/DNA ratio = 1 results in progressive H1 extraction, with DM and CM reaching a plateau up to ~90 and ~70% of total H1 content in the nucleus, respectively. These results are generally in agreement with the data in Fig. 2 and demonstrate that CM extracts from the nucleus always 20% less H1 than DM.

Histone H1 extraction at low DM or CM concentrations. It is clear that DM in low concentrations will be incorporated primarily in the most AT-rich sites to which it has the highest affinity. Figure 4 shows that the first ~10% of histone H1 above control is extracted at DM/DNA ~ 0.01. The DM (0.01) curve in Fig. 2 shows that this additionally extracted H1 is not removed from chromatin by PG only, because at any value of PG/DNA ≥ 6 the yield of H1 is about 10% higher than the amount extracted by PG only.

Analogous fraction (in contrast to work [13]) was revealed at a low CM concentration (CM/DNA = 0.01). Like in the case of DM, PG at all concentrations at CM/DNA = 0.01 extracts approximately the same additional amount of H1 (10-15%; Fig. 2, curve CM (0.01)).

Thus, the low concentrations of DM and CM influence on specific H1 fractions strongly bound in the AT- and GC-rich linkers, respectively.

For elucidation of the effect of chromatin compaction on extraction of this H1 fraction, we compared H1 yields from nuclei with unfolded chromatin structure and from initial condensed nuclei.

It was shown that if the nuclei were first treated with PG to extract 25% of total H1 (Fig. 5, PG (S1)) and, therefore, to unfold the supranucleosome chromatin structure, and then the DM portion (DM/DNA = 0.01) interacting with AT-linkers only was added to the second extraction, the extracted H1 amount is higher by 20% than in the control (Fig. 5, PG (S2)). If the same portion of DM was added to condensed nuclei prior to the first

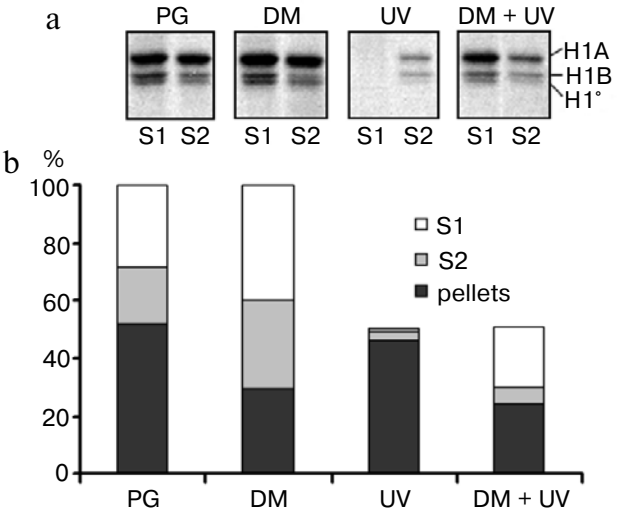

Fig. 5. Influence of low DM concentration (DM/DNA = 0.01) and UV irradiation on histone H1 extraction by polyglutamic acid (PG/DNA = 6). a) Electrophoregrams of H1 histone from the first (S1) and second (S2) supernatants. H1A, H1B, and H1° are the bands of histone H1. b) Diagram of the relative content of histone H1 in supernatants (S1, S2) and pellets. X-axis (here and in Fig. 6), treatment before the first PG extraction (PG, control; DM, treatment with DM; UV, UV irradiation; DM + UV, treatment with DM followed by UV irradiation). Y-axis, share of histone H1 in nuclear fractions, % of total nuclear content.

extraction, then the yield of H1 is only ~35% (Fig. 5, DM (S1)), i.e. only 10% higher than in the control. However, in this case the second extraction with the same portion of DM yields already 30% of total H1 (Fig. 5, DM (S2)) instead of 20% in the control (Fig. 5, PG (S2)), i.e. it turns out that 10% of total H1 not removed during the first extraction is removed in the second one.

It can be supposed that about the half of most DM-sensitive H1 fraction has different localization and bond strength in the supranucleosomal chromatin structure and, therefore, is extracted only after preliminary PG-induced unfolding of chromatin.

Effects of DM and CM on extraction of histone H1 subtypes. It is known that histone H1 consists of a series of subtypes. Seven of them have been described in mouse somatic cells [31]. In SDS-PAGE, histone H1 migrates mainly as three major bands: upper (H1A), middle (H1B), and lower (H1°). Actually, in accordance with the nomenclature of Lindner et al. [32], the H1A band consists of subtypes H1a, b, d, and e, and the H1B band consists mainly of subtype H1c but may include some part of H1a; the H1° band is only subtype H1°. The nuclear distribution of H1 subtypes is heterogeneous. Immunocytochemical localization of H1 subtypes from bands H1A and H1B shows that the H1A histones are located both in the center and on the periphery of the nucleus, and the H1B histones are found only within the interphase nucleus [33]. H1° is related mainly to the diffuse chromatin of differentiated cells [34]. The FRAP method has shown a weak binding for H1a and c, an intermediate binding for H1° and H1b, and strong binding for H1d and e [9]. When the binding strength was estimated by the interaction between H1 and chromatin *in vitro*, it was found that the affinity of subtypes increased in the series of H1a < H1c, H1b < H1d, H1e, H1° [35].

We have analyzed the influence of DM and CM (antibiotic/DNA = 0.1) on extractability of H1 histone subtypes from nuclei by means of PG. The DM and CM treatment was shown to result in the greater extraction of subtypes H1A and H1B, respectively; H1° was more sensitive to DM than to CM, but a minor part of H1° was more strongly bound to chromatin and even in the presence of DM or CM accumulated in the precipitate. It was especially noticeable during the extraction with high-concentration PG (PG/DNA = 30) in the most tightly chromatin-bound H1 pool (table).

Thus, H1A and H1B are preferably bound to AT-rich and GC-rich sequences, respectively. This conforms to the data on the presence of H1A in peripheral heterochromatin regions containing more AT-sequences and on the presence of H1B in the nucleus center enriched in GC-containing euchromatin [33]. Localization of the H1° subtype on AT-sequences probably has a certain advantage, though some part of it is tightly bound irrespective of the DNA sequence.

The same regularities in DM-sensitivity of subtypes comprising the H1A and H1B bands were revealed at DM/DNA = 0.01 (Fig. 6, PG and DM).

Effect of UV irradiation on histone H1 extraction from isolated nuclei. UV radiation is extensively used for structural investigations of chromatin. UV irradiation results in formation of DNA–protein and protein–protein cross-links [36, 37]. Among histones, the effect of cross-linking is most pronounced for H1 and H3; under irradiation of cellular nuclei (i.e. under more native conditions), it exceeds twofold the effect observed for isolated chromatin [38]. However, in most of the studies cross-links usually imply decrease in extractability of the proteins by dissociating agents [38, 39]. The actual amount of DNA–protein complex isolated from chromatin is no more than several percent [40].

Figure 7 shows the dosage dependence of histone H1 extractability by acid (0.75 M $HC1O_4$) on irradiation time. The graph reaches a plateau in the region of 50% of total H1 in the nucleus after 20-30 min of irradiation. Soft DNA hydrolysis in the precipitates of irradiated nuclei (50°C, 15 min, and 0.75 M $HC1O_4$) did not increase the yield of histone H1 (not illustrated), demonstrating either the small number of H1–DNA cross-links or simultaneous cross-linking of H1 with DNA and with other components of the nucleus, primarily proteins. Thus, UV irradiation of the nuclei results in cross-linking of about half of histone H1 contained in the nucleus, probably due to protein–protein cross-links.

We studied the influence of UV irradiation of nuclei on H1 fractionation by PG and DM (Fig. 5). As a result of irradiation, the bands of PG-extracted histone H1 noticeably decreased to nearly complete absence of material on

Effects of DM and CM (antibiotic/DNA = 0.1) on the ratio of histone H1 subtypes (in % of total H1 in the preparation) in the pellets of nuclei extracted by PG (PG/DNA = 30); $X \pm \sigma$ (root mean square deviation)

Treatment	Share of H1 in nuclear pellets, %	H1A, %	H1B, %	H1°, %
PG	54.1 ± 4.6	65.3 ± 0.8	24.3 ± 1.2	10.4 ± 1.1
DM + PG	5.6 ± 1.3	51.0 ± 5.1	34.6 ± 2.2	15.4 ± 3.6
CM + PG	26.1 ± 2.4	63.4 ± 3.1	20.1 ± 2.5	16.5 ± 1.8

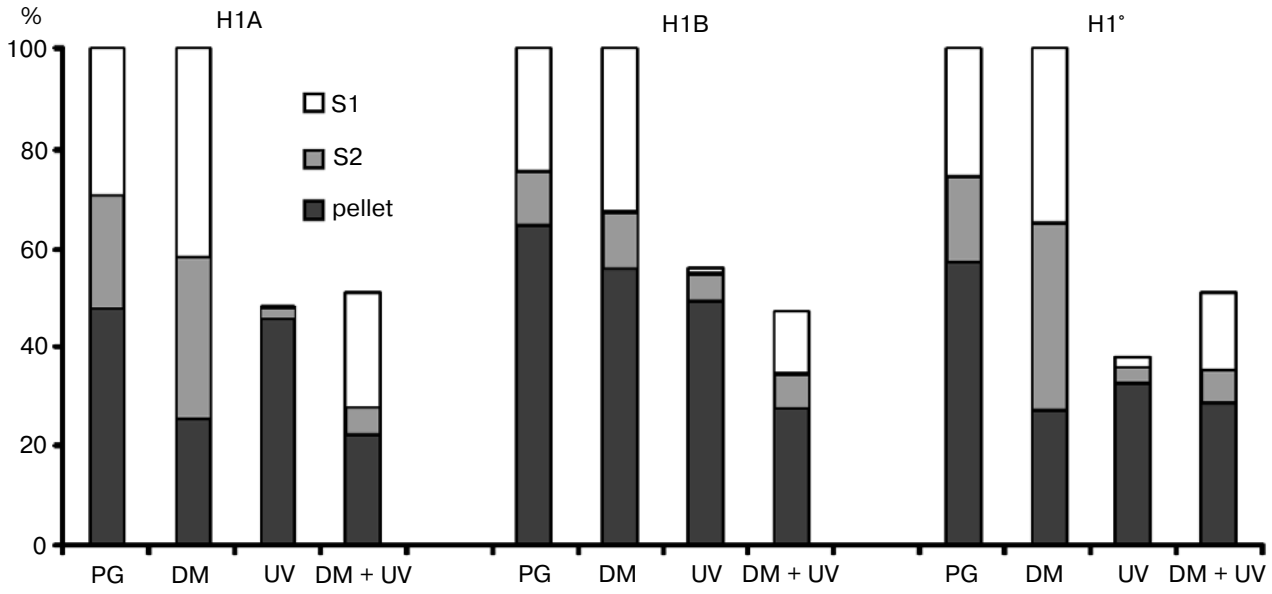

Fig. 6. Influence of low DM concentration (DM/DNA = 0.01) and UV-irradiation on protein content in bands of histone H1 (H1A, H1B, and H1°) in supernatants and pellets obtained by PG extraction of nuclei (PG/DNA = 6). Y-axis, protein share in bands of histone H1 (H1A, H1B, or H1°) in nuclear fractions, % of total H1 content in this fraction.

the electrophoregram (Fig. 5a; UV (S1)). Subsequent treatment of the pellet of these nuclei with PG + DM mixture extracts into solution only ~3% of total H1 (Fig. 5; UV (S2)) instead of 20% in the control (Fig. 5; PG (S2)). At the same time, still ~45% of total H1 can be extracted from the nuclear pellet by SDS or $HClO_4$.

Previously we have shown that UV radiation stabilizes the structure of condensed chromatin regions in the interphase nucleus, decreasing the extraction of total proteins and DNA from the nucleus [41]. Results of the present work show that the UV radiation-induced chromatin stabilization leads not only to the cross-linking of ~50% of total H1 but also to blocking of the major portion of remaining H1 in cross-linked chromatin fibril, preventing its extraction by PG-type agents. Histone H1 enclosed in the network can be found in the pellet under the action of a more severe agent, e.g. SDS or $HClO_4$ destroying the entire chromatin structure or the secondary structure of DNA. Such stabilization of chromatin structure was also revealed as a result of irradiation of nuclei by visible light in the presence of photosensitizers (methylene blue [42] and ethidium bromide [43]).

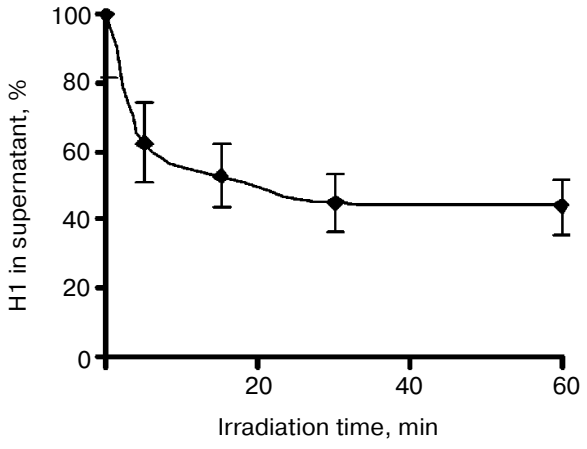

Fig. 7. Influence of UV irradiation on histone H1 extraction from nuclei by 0.75 M perchloric acid. The average measuring error was calculated from the results of three experiments.

Since after the cross-linking effect of UV light and repeated PG treatment in the presence of DM (DM/DNA = 0.01) it is still possible to isolate 3-4% of total H1 from the nuclei (Fig. 5a; UV (S2)), it can be concluded that this H1 fraction is neither cross-linked nor enclosed in the network, being a part of the most DM-sensitive histone H1 fraction.

DM treatment of the nuclei before UV irradiation abruptly increases the yield of histone H1 during the first PG extraction (up to 20% of total nuclear H1) (Fig. 5; DM + UV (S1)). The repeated treatment of such nuclei with PG + DM adds to the extraction of ~5% of the nuclear H1 (Fig. 5; DM + UV (S2)). However, both extractions do not change the quantity of tightly cross-linked histone H1 (~50%). The diagram (Fig. 5b) also shows both for UV irradiated and not irradiated nuclei (DM + UV and DM diagrams, respectively) that twofold DM treatment equally enhances H1 extraction compared to a single treatment (UV and PG diagrams, respectively). However, just the first portion of DM under UV irradiation extracts 20% of total H1, which is probably most quantity of H1 undergoing its action.

Naturally, the H1 fraction localized on AT-rich linkers (up to 25% of total H1) must react first to low DM doses. Consequently, the larger part of this fraction is affected at DM/DNA = 0.01. Based on the above, we suggest the existence of a most sensitive to DM special H1 fraction amounting to about one fourth of the total nuclear H1. This fraction, although falls within the network of UV-stabilized chromatin, is not tightly cross-linked, and 3-4% of its total H1 is not stabilized by UV irradiation. It is not inconceivable that these 3-4% of total nuclear H1 can have specific localization, for example in the SAR/MAR-regions.

Effect of UV irradiation on extraction of H1 subtypes. Figure 6 shows that, as a result of UV irradiation, each of the electrophoretic H1 bands decreases approximately by half, apparently due to formation of protein–protein cross-links. At the same time, the cross-linking of H1 subtypes shows only a minor difference, slightly increasing in the series H1° > H1A > H1B (UV).

DM treatment of nuclei prior to UV irradiation enhances the extractability of H1 subtypes as consistent with the DM sensitivity without irradiation (Fig. 6; DM), i.e. H1A > H1° > H1B (Fig. 6; DM + UV (series S1)). After irradiation, the subtypes of the H1B and H1° bands are more extracted in the presence of DM (series S2). Consequently, H1A is most difficult to extract from what has been fixed in the pellet, in spite of its higher DM sensitivity, probably due to its location in more sterically closed spaces of the chromatin network.

DISCUSSION

The study of effects of various factors (antibiotics, UV radiation, etc.) on histone H1 extraction by PG from rat liver nuclei leads to the following conclusions:

– the high-polymer anion PG extracts histone H1 from the nuclei in the same way as from isolated chromatin [17]. However, electron microscopy of nuclear preparations has shown that PG treatment does not result in complete unfolding of chromatin to the state of elongated fibril as beads on a string. Supranucleosomal structure is partially decondensed to the state when 10-nm fibrils can already be discerned, however retaining some elements of packing of the supranucleosomal level as ring or half ring structures;

– DM and CM destabilize the chromatin structure in the nucleus without changing it. This destabilization is demonstrated by enhanced H1 extraction by PG. We have estimated that ~25% of the nuclear histone H1, being a part of tightly bound H1 (not removed by PG), is extracted only in the presence of DM (AT-rich regions) and 5% is extracted only in the presence of CM (GC-rich regions). The data conform to those presented in the literature on enrichment of linkers with AT-rich sequences [27, 28]. The found 20% difference is maintained over a wide range of PG concentrations (PG/DNA = 6-60; Fig. 2, curves DM and CM) and may be evidence of reliability of the size of H1 fractions associated with the respective sequences. One should emphasize the significance of choosing a dissociative agent (in particular, PG), relatively weak for revealing H1 fractions with different sensitivity to the antibiotics that reduce the strength of interaction;

– there are differences in H1 extraction from the condensed state of chromatin compared to the relaxed state. This concerns the H1 fraction associated with AT-rich linkers and released by PG at a low DM concentration (DM/DNA = 0.01). Our experiments with different orders of PG and DM treatment of nuclei (the diagram in Fig. 5) have shown that half of this H1 fraction is not extracted under the influence of DM on condensed chromatin but is extracted from relaxed chromatin. The observed differences in H1 extraction can be explained by assuming that DM modifications of condensed and partially unfolded chromatin proceed differently, or the yield of H1 will be affected by not only modifications but also the process of unfolding of the modified condensed chromatin. At that, the structures formed in the course of unfolding in the presence and absence of DM may be different, even if their electron-microscopic patterns look similar. The latter assumption seems to be preferable;

– although H1 subtypes significantly differ in the strength of interaction with chromatin both *in vivo* and *in vitro* [9, 35], no enrichment with any of the subtypes has been noted during PG extraction. Nevertheless, there are minor differences in the sensitivity of subtypes to various antibiotics, but the more tightly bound (according to literature data) subtype H1° is accumulated in the precipitate;

– ~50% of total H1 can be tightly fixed in the nucleus (supposedly due to protein–protein cross-links) by means of UV irradiation. According to our preliminary data, there is no such fixation in unfolded chromatin. It is also found that H1 bound to AT-linkers is not covalently cross-linked to DNA or proteins but retained in the chromatin network formed during UV irradiation, or is not included into this network at all if the nuclei have been treated with DM prior to UV irradiation.

Histone H1 is responsible for the supranucleosomal organization of chromatin; hence, it was most interesting to understand how the revealed H1 fractions may be related to this organization.

The question about supranucleosomal chromatin organization in the nucleus is still open. Most the studies in this field have been performed *in vitro* on nucleoprotein reconstructed from DNA and histones or after re-condensation of pre-unfolded chromatin. Based on these experimental data, the helical packing of nucleosome fibril with zigzag folding in a one-row or two-row variant has been recognized as a principle of formation of supranucleosomal structure [44]. However, one should remember that the DNA fragments of limited length used in the

experiments on reconstruction have free ends, in contrast to the situation in the nucleus where the ends of fibrils are fixed. The parameters of initial chromatin structure are lost during the unfolding of condensed chromatin and, as it seems, not restored during its refolding [45, 46]. Thus, the conclusions based on these model experiments *in vitro* may not conform to the real situation in the nucleus. Also known is the existence of a model of discrete structure of a supranucleosomal fibril consisting of subunits (nucleomers [47] or superbeads [48]) confirmed by electron microscopy and biochemistry [49, 50]. If the structure of supranucleosomal chromatin fibril is discrete, the division of structural monomers (elementary units) of this fibril must be accompanied by regular repetition of the conformation of linkers and the interactions between H1 and other structural proteins along the fibril length, thereby forming H1 fractions differing in interaction within the chromatin structure. Certainly, the presence of other proteins in the closest environment of histone H1 may enhance the spread in the interaction between histone H1 molecules, slightly smoothing the differences between the fractions.

When analyzing the results, we took notice of multiplication (probably random) factor 2 of the quantitative sizes of the fractions. In this connection, we attempted to correlate (merely hypothetically) the quantitative data on H1 fractions that we had obtained with ones of the principles of structural organization of chromatin, namely, discreteness of supranucleosomal levels.

The hypothesis was based on two assumptions: the revealed H1 fractions are related to the supranucleosomal structure and uniformly distributed over chromatin.

Since all histone H1 in the nucleus can be divided by UV irradiation into two approximately equal fractions (cross-linked and not cross-linked), it can be envisaged that H1 molecules in every second nucleosome may be different in the parameters of spatial location and, probably, the tightness of binding. Such situation is possible in the interphase nucleus. Preferable output of dinucleosomes in the course of nuclease treatment of the nuclei under certain ionic conditions [51, 52] or in the presence of Triton X-100 has been reported [53]. However, such division of H1 is unlikely in a fibril with solenoid packing of nucleosomes, where all linkers are identically localized inside the fibril by definition.

As a result of using the polyanion, for which the interaction with outwardly located H1 histones seems to be preferable, the H1 pool was also approximately on two equal fractions. However, this result may be concerned with the dissociating ability of PG specifically used in our studies.

We have also revealed that ~25% of the nuclear H1 is removed by PG only in the presence of DM but not CM, i.e. one fourth of histone H1 molecules is tightly bound just in the AT-rich linkers. Approximately the same is the share of H1 fraction that is removed by DM at low concentrations (i.e. also bound in AT-linkers) from the chromatin network formed during exposure to UV radiation. It seems that what is meant here is the same fraction of histone H1. It can be supposed that molecules of this fraction are located, accordingly, on every forth nucleosome of the chromatin. The preferable output of tetranucleosomes together with di- or octanucleosomes was actually observed during nuclease hydrolysis of chromatin in nuclei [51]. Note that the nucleomer model suggests two turns of the nucleosome fibril by four nucleosomes in each [50].

As we have supposed above, the H1 fractions under discussion are evenly distributed over the chromatin; however, it is known that many AT-rich sequences are located in clusters, e.g. in satellite sequences of the centromeric region of chromosomes. It was also shown that random DNA fragments, $2 \cdot 10^5$-$2 \cdot 10^6$ Da in length, include at least one site of preferable binding for H1 [54], while AT-rich sequences are just such points of binding for H1. Note that 25% of AT-linkers make up ~5% of rat liver nuclear DNA, and this quantity may be only a part of all AT-rich sequences and, quite probably, are uniformly distributed in chromatin.

We have found that half of the H1 fraction bound to AT-rich linkers, which makes up to ~1/8 of the nuclear histone H1, is not extracted from condensed chromatin under our conditions but is extracted from relaxed chromatin. According to the data of Kiryanov et al. [50] and Stratling et al. [49], the nucleomer (superbead) of rat chromatin consists of eight nucleosomes (see also [39] and [55]). It can be supposed that every eighth nucleosome will contain H1 characterized by its own way of interaction with chromatin, e.g. on the boundary of each nucleomer (superbead).

Some of the revealed small fractions (the fraction sensitive to CM but insensitive to DM (~5% of the nuclear H1); the fraction extracted in the presence of DM that is not at all affected by UV radiation (~3%); and the fraction resistant to both antibiotics (~3%)) may determine the boundaries of larger structural formations consisting, e.g. of two (approximately corresponding to a rosette loop [46]) or four nucleomers (superbeads).

As a whole we believe that there is a correlation for rat liver chromatin between the number of nucleosomes in the known discrete supranucleosomal chromatin formations and in the revealed division of total H1 pool into fractions. This is probably not accidental but reflects the types of interaction between different H1 fractions responsible for certain periodical location of nucleosomes in the structure during the folding of nucleosome fibril into more highly organized chromatin structures.

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