NUCLEOSOME STRUCTURE STUDIED WITH PURIFIED ANTIBODIES TO HISTONES H2B, H3 AND H4

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1. Introduction

The basic structure of chromatin appears to consist of a string of repeating units called nucleosomes [1] which contain two molecules each of the histones H2A, H2B, H3 and H4 and about 200 base pairs of DNA [2–5]. Nucleosomes which can be obtained by brief digestion of chromatin with micrococcal nuclease, have an average diameter of 1[illegible] Å and sediment at 11 S in isokinetic density gradien[illegible] [6].

The availability of specific antisera to the different histones raises the possibility of studying the topography of the nucleosome by immunochemical methods [7]. If the antigenic determinant regions of histones could be located [8–10] and the corresponding antibody fractions prepared, it may become possible to determine the precise orientation of particular histones within the nucleosome. We now report on the location of one antigenic determinant in histone H2B of calf thymus, and illustrate the use of purified H2B, H3 and H4 antibodies for elucidating the surface structure of nucleosomes.

2. Experimental

Calf thymus histones H2B and H4 were isolated as described [11]. H2B was further purified on a 100 × 1.6 cm Sephadex G-100 column. Various fragments of H2B were obtained by cleavage with dilute HCl [12], cyanogen bromide [13] and *Staphylococcus aureus* protease [14] and were purified by gel filtration and CM cellulose chromatography. Histone H3 from chicken erythrocytes was purified as described [15]. The purity of the histones and H2B fragments was ascertained by polyacrylamide gel electrophoresis [16] using 50–100 μg samples, as well as by N-terminal analysis [17].

Antibodies to H2B, H3 and H4 were obtained in rabbits [18]. Complement fixation tests were performed as described [19]. The antigenic activity of H2B fragments was tested by determining the ability of the peptides to inhibit complement fixation [9]. Specific H2B and H3 antibodies were isolated from antisera, using columns of CH–Sepharose 4B (Pharmacia, Uppsala) to which the homologous histones were coupled, at pH 4.5, with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Merck, Darmstadt) [20]. Specific H4 antibodies were purified on a column of AH–Sepharose 4B to which H4 had been coupled. Antibodies were eluted from the immunoadsorbent columns with 0.1 M glycine–HCl buffer, pH 2.8, containing 0.5 M NaCl, dialysed against phosphate buffered saline pH 7.2 and concentrated to the original volume of antiserum.

Nucleosomes were prepared from rat liver nuclei by digestion with 150 units/ml micrococcal nuclease (Boehringer, Mannheim) using 6 min incubation at 37°C [6]. Digested chromatin (0.5 ml containing 7.0 A_{260} units) was layered on isokinetic sucrose gradients [21] which were then centrifuged for 27 h at 27 000 rev/min at 4°C in a Beckman SW27 rotor [22]. Fractions were collected by upward displacement of the contents of centrifuge tubes and monitored by measuring A_{254} in an ISCO model 640 fractionator (ISCO, Lincoln, Nebraska). The nucleosome monomer fraction [6] was collected and dialysed overnight against 1 mM EDTA, pH 7.0 [22]. Purified nucleo-

somes were allowed to react with preparations of the various histone antibodies and the mixtures were centrifuged for 27 h at 27 000 rev/min using the same isokinetic gradients as before.

3. Results and discussion

Complement fixation results obtained with anti-H2B and anti-H3 sera used in this work are shown in fig.1. The specificity of the antisera to each histone is demonstrated by the absence of any cross-reactivity with the other two histone antigens. By comparing the ability of various cleaved peptides of H2B to inhibit complement fixation in the H2B-anti H2B system, it was found that the region corresponding to residues 36–50 possessed considerable antigenic activity. This peptide was used to prepare an immunoadsorbent column.

The activity of specific antibody fractions eluted from the immunoadsorbent columns was determined by complement fixation tests. The results presented in fig.2 indicate that all the anti-histone antibodies present in the different antisera were able to bind to

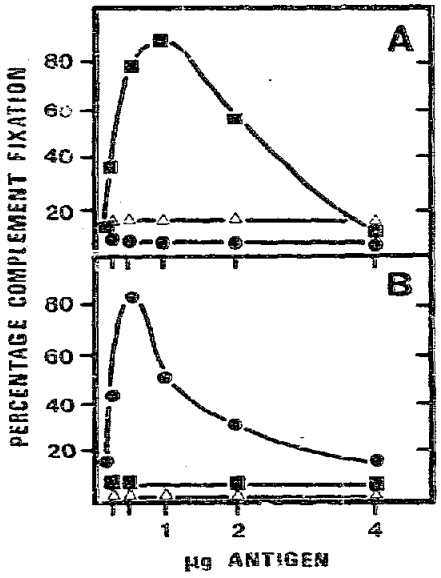

Fig.1. Complement fixation of anti-histone sera with homologous and heterologous histones: (■–■) H2B; (△–△) H4; (●–●) H3. (A) Anti-H2B serum diluted 1:300-fold. (B) Anti-H3 serum diluted 300-fold.

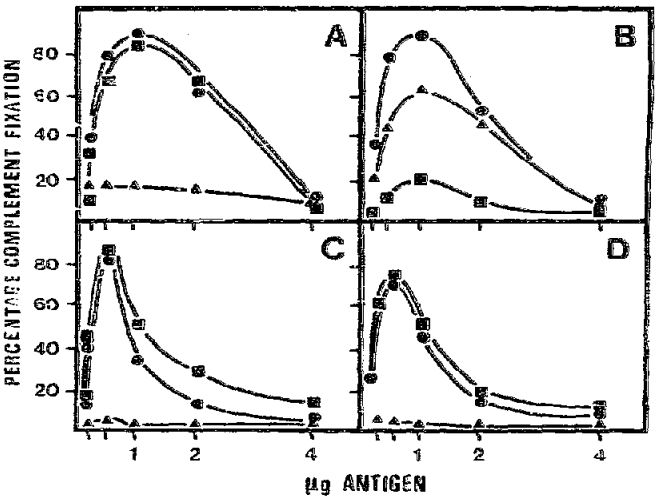

Fig.2. Complement fixation of purified anti-histone antibodies with homologous histones: (●–●) Original antisera prior to immunoadsorption; (■–■) Antibody fractions purified by immunoadsorption; (▲–▲) Fractions of antisera which did not bind to the Sepharose column. (A): (●–●) Anti-H2B serum diluted 1:600; (■–■) anti-H2B antibody, 1:250; (▲–▲) non-specific fraction from anti-H2B serum. The antigen was the homologous histone H2B. (B): (●–●) Anti-H2B serum 1:600; (■–■) antibodies, 1:250, absorbed on H2B peptide 36-50 attached to Sepharose column and eluted, at pH 2.8. (C): (●–●) Anti-H3 serum, 1:300; (■–■) anti-H3 antibody, 1:100. (D): (●–●) Anti-H4 serum, 1:500; (■–■) anti-H4 antibody, 1:150.

the homologous histone–Sepharose columns. The total antibody activity that could be eluted from the various columns, at pH 2.8, represents about 60% of the activity that was present in the original antisera (fig.2). In the case of the immunoadsorbent column prepared with H2B peptide 36–50 (fig.2B), only a small fraction of the total H2B antibodies was bound and could be eluted, at pH 2.8. The activity of the antibodies purified by immunoadsorption was also checked by recycling a small fraction of the purified antibody a second time through the immunoadsorbent columns. It was found that 40–50% of the soluble material present in these antibody preparations was no longer capable of binding to histones. Presumably this is due to denaturation of IgG on exposure to acid pH [23,24].

Following density gradient fractionation of the rat liver chromatin digest, a regular series of five

resolved peaks was obtained (fig.3A). Similar patterns have been described [6,22] which showed that the peaks correspond to nucleosome monomers, dimers, trimers etc. When the monomer fraction was collected and submitted to a second cycle of gradient centrifugation no contamination with any polymers was visible (fig.3B). All nucleosome preparations used in subsequent binding tests with antibodies showed sedimentation patterns similar to that of fig.3B.

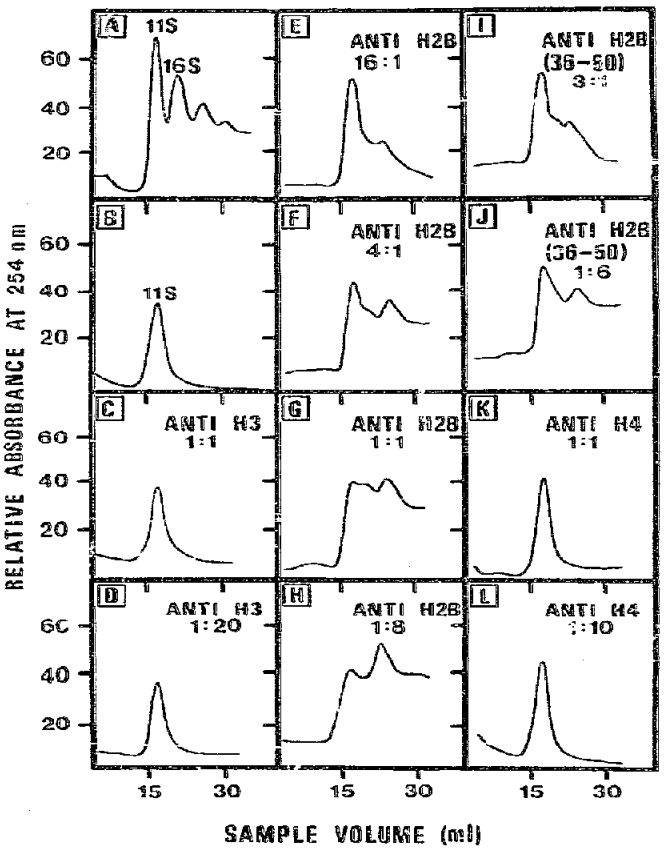

Fig.3. Isokinetic sucrose gradient analysis of nucleosome preparations, following incubation with purified anti-histone antibodies. The molar ratio of nucleosomes to antibody in the incubation mixtures is indicated on each diagram. Samples (0.5 ml) were layered on 38 ml tubes and centrifuged for 27 h at 27 000 rev/min at 4°C. (A) Digested chromatin (0.5 ml containing 7.0 A_{260} units) showing nucleosome monomers (11 S), dimers (16 S), trimers etc. (B) Purified nucleosome monomers (1.8 A_{260} units). (C) Nucleosomes + H3 antibodies. (D) H3 antibodies. (E) H2B antibodies. (F) H2B antibodies. (G) H2B antibodies. (H) H2B antibodies. (I) H2B (peptide 36–50) antibodies. (J) H2B (peptide 36–50) antibodies. (K) H4 antibodies. (L) H4 antibodies.

To study their antigenic properties purified nucleosomes were mixed in various molar ratios with different antibody preparations and were incubated at 37°C for 30 min. The mixtures were then submitted to gradient centrifugation and the results are presented in fig.3C–L.

When used in nucleosome to antibody molar ratios of 1:1, 1:10 and 1:20, purified H3 and H4 antibodies did not affect the nucleosome sedimentation pattern (fig.3C,D,K,L). The inability of the H3 and H4 antibodies to bind to the surface of nucleosomes was confirmed when total anti-H3 and anti-H4 globulins (prepared by precipitation from antiserum with ammonium sulphate) were used instead of purified histone antibodies. These globulins were also found not to affect the nucleosome sedimentation pattern.

Since the histone antibodies were obtained from animals immunized with histone–RNA complexes [18], it could be argued that some antibodies to conformational determinants present in such complexes cannot be absorbed by Sepharose–histone columns and cannot react with uncomplexed histone molecules. The negative results obtained with total H3 and H4 globulins indicate, however, that the absence of binding to the nucleosome surface is probably not caused by conformational changes related to nucleic acid binding. On the other hand the possibility cannot be excluded that the quaternary structure arising from histone–histone interaction within the nucleosome generates novel conformational determinants absent in the histone monomers. Convincing evidence exists for the presence of such new determinants or neotopes in the quaternary structure of viral capsids [25–28]. It is also possible that the absence of reactive antigenic determinants of H3 and H4 at the surface of nucleosomes reflects the central position of the arginine-rich histones in a 'kernel' surrounded by DNA coils [29,30].

Anti-H2B antibodies were found to markedly affect the sedimentation pattern of nucleosomes. At a nucleosome to antibody molar ratio of 16:1 (i.e., with 320 times less antibody than was used in fig.3D) a second peak caused by nucleosome dimers bridged through one IgG molecule was visible (fig.3E). This peak has a sedimentation rate intermediate between

nucleosomes dimers and trimers (fig.3A). With increasing quantities of anti-H2B antibody, the dimer peak enlarges (fig.3F–H). At molar ratios of 4:1 and 1:1, a shoulder of 13 S is visible, which probably corresponds to a single monomer–IgG complex. Anti-H2B globulins affected the nucleosome sedimentation pattern in the same way as purified H2B antibodies.

Antibodies specific for peptide 36–50 of H2B were also found to bind to nucleosomes (fig.3I,J). This suggests that this region of the H2B molecule is situated at the nucleosome surface and is not involved in DNA–histone or histone–histone interactions.

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