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# Chromatin Subunits Elicit Species-Specific Antibodies against Nucleoprotein Antigenic Determinants†

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ABSTRACT: Nucleosomes composed of 195 base pairs of DNA associated with histones H2A, H2B, H3, and H4 purified from chicken erythrocyte nuclei were used to elicit antibodies in rabbits. Specific serological reaction between the antisera and the nucleosomes is demonstrated by immunodiffusion, immunofluorescence, microcomplement fixation, solid-phase radioimmunoassay, immunosedimentation, and polyacrylamide gel electrophoresis of 5′-$^{32}$P end-labeled nucleosomes. The antisera did not react with DNA extracted from these nucleosomes, core histones, or the cross-linked histone octamer from chicken erythrocytes, calf thymus total histones, or chromosomal proteins HMG-1 or HMG-17. Nucleosome antigenicity was not affected by redigestion with micrococcal nuclease. Digestion with DNase I brought about 50% loss of reactivity while digestion with trypsin or proteinase K resulted in total loss of activity. The antisera reacted strongly with trimer, dimer, and monomer nucleosomes as well as with the core particle (145 base pairs of DNA) and subnucleosome (<145 base pairs) obtained from chicken. It reacted less well with nucleosomes obtained from HeLa cells and was almost totally devoid of activity against chromatin particles obtained from rat liver or wheat germ. Experiments employing the technique of transferring proteins from a polyacrylamide gel to diazobenzyloxymethyl paper and visualization of antigens by autoradiography excluded the possibility that the serum contains antibodies against tissue-specific antigens which are found in small amounts but are very immunogenic. It is concluded that most of the antibodies in the sera are directed against nucleoprotein antigenic determinants composed of the N-terminal portion of the histones and segments of DNA. Antibody binding is dependent on contact between the histone and DNA segments and is independent of the integrity of the entire nucleosome. Thus, certain histone DNA contacts remain intact even though the structure of the nucleosome has been disrupted.

Higher order chromatin structures are built up from linear chains of basic deoxyribonucleoprotein repeating unit (the nucleosome) whose distribution and structure are ubiquitous. [See reviews by Kornberg (1977) and Felsenfeld (1978) for details on chromatin structure.]

Though several criteria have been used to identify nucleosomes, none of these criteria by themselves can unequivocally distinguish between intact and damaged or incomplete particles. Thus, a complex of the arginine-rich histones H3 and H4 with DNA of appropriate length will generate low-angle X-ray diffraction patterns, electron microscopic images, micrococcal nuclease and DNase I digestion patterns, and torsional constraints in SV40 DNA typical of intact particles (Moss et al., 1977; Oudet et al., 1978; Camarini-Otero et al., 1976; Sollner-Webb et al., 1976; Bina-Stein & Simpson, 1977). Torsional constraints in SV40 DNA, nucleosome-like electron microscopic images, and DNase I digestion patterns have been obtained also with DNA which is not complexed with histones (Eickbush & Moudrianakis, 1978; Griffith, 1976; Liu & Wang, 1978).

In an effort to generate a convenient and sensitive test which could be used as a criterion for chromatin particles, we immunized rabbits with chicken erythrocyte nucleosomes which have been stripped of nonhistone proteins and histones H1 and H5. In this manuscript we present evidence that the nucleosomes elicited specific antisera and identify the nucleosome antigenic sites.

## Materials and Methods

*Preparation of Antigens and Antisera.* (*a*) *Nucleosomes.* Chicken erythrocyte and rat liver nuclei were prepared by established methods (Axel et al., 1973; Goldblatt & Bustin, 1975). Wheat germ nuclei were prepared according to Luthe & Quatrano (1980). Chicken, rat liver, and wheat germ nuclei were washed with 0.6 M NaCl, swollen in $H_2O$, made 0.7 mM in $CaCl_2$ and 10 mM in Tris-HCl, pH 7.5, digested with micrococcal nuclease [100 units/(mL mg of DNA), 37 °C, 4–15 min]. The reaction was terminated by addition of EDTA to 2 mM. After dialysis the digest was fractionated on a 12.5–45% isokinetic sucrose gradient in a Beckman Ti 14 zonal rotor (McCarthy et al., 1968; Varshavsky et al., 1976). HeLa nucleosomes were prepared as described by Whitlock & Simpson (1976).

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Antisera were elicited by injecting rabbits at multiple intradermal sites with 1 mg of nucleosome DNA in 66% Freund's complete adjuvant. Booster injections were administered on 3 subsequent weeks followed by a final intravenous injection. The rabbits were bled weekly starting 1 week after the final administration of the antigen.

(*b*) *Histones, Nonhistone Proteins, and DNA.* Characterization and preparation of calf thymus histones, HMG proteins, and their antisera have been described elsewhere (Goldblatt & Bustin, 1975; Romani et al., 1980). Purified chicken erythrocyte nucleosomal DNA was extracted by using a modified Marmur procedure (Britten et al., 1974). Cross-linked nucleosomes and histone octamers were gifts of Dr. A. Stein; actin was a gift from Dr. P. D. Chantler; micrococcal nuclease was from Worthington (NFCP grade). Acid-extracted chicken histone was fractionated on a 80 × 1.1 cm Sephacryl S-200 column in 20 mM HCl, 50 mM NaCl, and 0.02% $NaN_3$. H2A and H2B were further purified according to Oliver et al. (1972).

*Antisera Purification.* IgG was prepared from crude heat-inactivated serum by DEAE-cellulose chromatography (Bustin et al., 1977b). Anti-histone H5 activity was removed from antinucleosome serum by adsorption on a Sepharose 4B-H5 column.

*Microcomplement Fixation.* Microcomplement fixation was performed according to Wasserman & Levine (1961) with minor modifications (Stollar & Ward, 1970).

*Solid-Phase Radioimmunoassay.* Solid-phase radioimmunoassay was carried out as previously described (Romani et al., 1980), except that Protein A was iodinated with Bolton and Hunter reagent (Bolton & Hunter, 1973).

*Phosphorylation on Nucleosomal DNA.* End labeling of nucleosomes was carried out as described (Simpson & Whitlock, 1976).

*Immunofluorescence.* Fresh blood was collected from the wing vein of white Leghorn cocks. Smears were prepared on glass slides, dried in air, and fixed for 5 min in a methanol bath packed in dry ice. The following reaction sequence was used: (i) 30-min incubation at 37 °C with 50 μL of 10 mg/mL BSA; (ii) 30-min washing in PBS at 4 °C (two changes each of 500 mL); (iii) 5 h, 37 °C, with either (a) 50 μL of DEAE and Sepharose 4B-H5 column purified antiserum (1:100 dilution) or (b) normal rabbit serum (1:100 dilution); (iv) washed 12 h, 4 °C, in PBS (two changes, 500 mL each); (v) incubated 1 h, 37 °C, with 50 μL of FITC-labeled Protein A; (vi) washed 3 h, 4 °C, in PBS (two changes); (vii) mounted in AquaMount and photographed in a Zeiss Photomicroscope III.

*Electrophoresis and Transfer to Paper.* Whole particles and DNA were fractionated by using neutral Loening (1967) polyacrylamide gels.

Proteins were fractionated by using the discontinuous sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel system of LeStourgeon & Rusch (1973). When gels were tranferred to paper, DATD was used as a cross-linker at a concentration of 20:1 acrylamide/DATD. Transfer and subsequent antibody and Protein A treatment were effected according to Renart et al. (1979).

*Analytical Procedures.* Protein concentration was measured by using the procedure of Lowry et al. (1951) or by using Bio-Rad Protein Assay Reagent. DNA concentration was estimated by using 1% $A_{260}$ = 200.

## Results

*Characterization of the Antigen.* Purified nucleosomes were obtained from chicken erythrocyte chromatin which was digested with micrococcal nuclease and fractionated on a sucrose gradient containing 0.6 M NaCl. The size of the DNA and the protein content of the chromatin particle used to immunize rabbits are presented in Figure 1. The average particle contained 195 base pairs of DNA complexed with the core histones H2A, H2B, H3, and H4. Protein gels were overloaded with these histones but showed no trace of other proteins. Thus, the antigen consists of a chromatin core particle to which a short piece of DNA, equivalent to the length of the linker region between two chromatin core particles, was attached. It is devoid of detectable amounts of histones H1 and H5 or nonhistone chromosomal proteins.

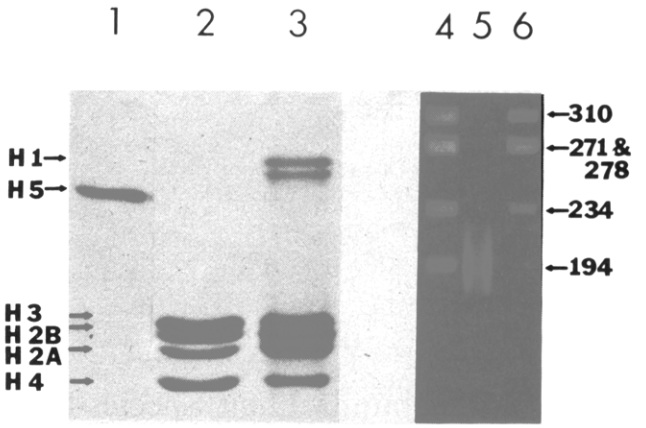


FIGURE 1: Characterization of the protein and nucleic acid content of the immunogen. Lanes 1–3 characterize the proteins on $NaDodSO_4$–polyacrylamide gels. Lanes 4–6 characterize the DNA by electrophoresis on 3.75% polyacrylamide gels. Lanes 1–6 depict respectively histone H5, proteins from the immunogen, calf thymus histones, $\phi$X174 standard, DNA of the immunogen, and $\phi$X174 standard.

*Detection of Specific Binding of Antinucleosome Sera to Nucleosomes.* Nucleosome–antinucleosome interaction was demonstrated and measured by several serological assays.

(*a*) *Immunodiffusion.* A single precipitin band is obtained when nucleosomes are tested against various concentrations of the antisera (not shown). The thickness and the position of the band change with sera dilutions. At high sera concentrations a heavy band relatively close to the center well, containing nucleosomes, is observed, while at a lower concentration the band is fainter and closer to the peripheral wells which contain antisera. Visible bands were obtained only at a nucleosome concentration of 0.5 mg/mL or higher. Diffusion against control sera did not produce a precipitin band.

(*b*) *Indirect Immunofluorescence.* The location of the antigen in erythroid cells was visualized by indirect immunofluorescence. The photomicrographs presented in Figure 2 reveal that the antigens are localized exclusively in the nucleus of the cell. Note that the fluorescence intensity of the diffuse nucleus present in the reticulocyte was significantly higher than that observed in the compact, dense nucleus of the mature erythrocytes. This situation probably reflects hindrance to antibody penetration into the more compact nucleus. In both cell types the fluorescence is confined to the nucleus of the cell and is absent from the cytoplasm.

(*c*) *Microcomplement Fixation.* The interaction of nucleosomes with antinucleosome sera results in a complement binding complex. This interaction will still be detectable at sera dilutions of 1:6000 (see Figure 3). Addition of control sera to nucleosomes did not result in complement-fixing complexes.

(*d*) *Solid-Phase Radioimmunoassay.* The most sensitive, convenient, and accurate way to detect and quantitate the

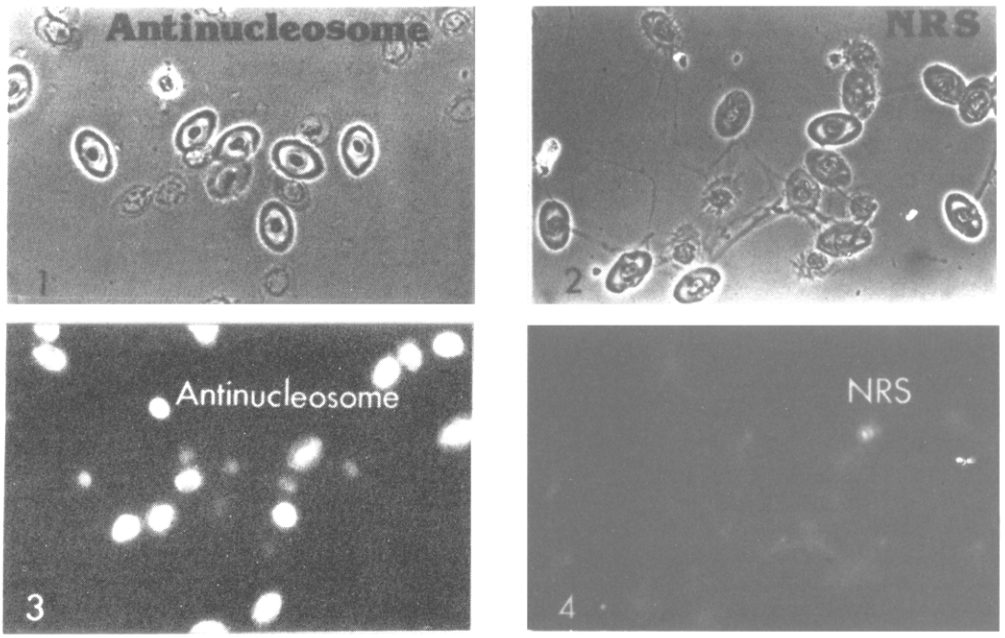


FIGURE 2: Binding of antinucleosome to erythroid nuclei demonstrated by immunofluorescence. Indirect immunofluorescence with purified antinucleosome IgG (3) and normal rabbit serum (4) at a dilution of 1:100. 1 and 2 phase-contrast photomicrographs correspond to 3 and 4.

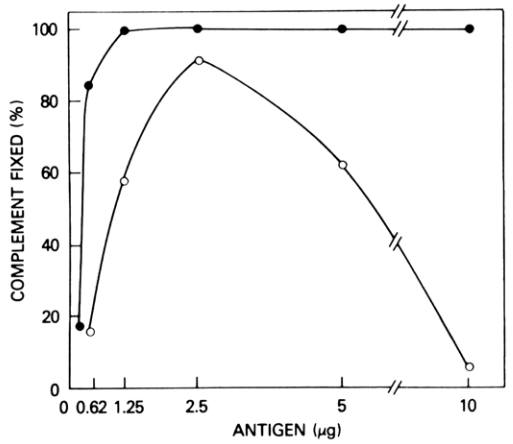


FIGURE 3: Complement fixation curves of antinucleosome sera treated with nucleosomes. Complement fixing activity of antinucleosome serum diluted 1:3000 (●) and 1:6000 (○).

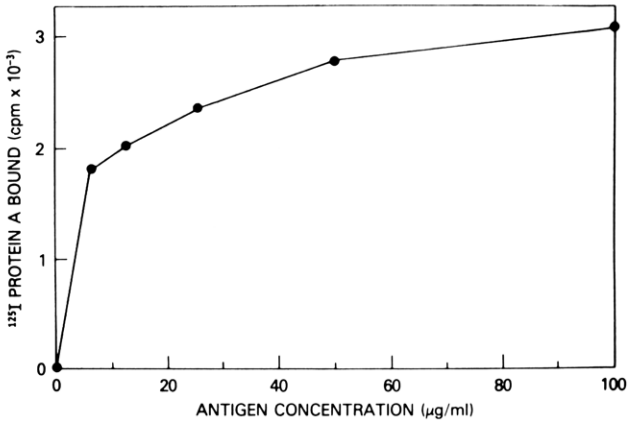


FIGURE 4: Dependence of antibody binding on nucleosome concentration determined by solid-phase radioimmunoassay. 50 μL of antigen solution at each concentration was added to individual microtiter plate wells. Antiserum was added at a 1:100 dilution.

interaction between nucleosomes and antinucleosome sera is the solid-phase radioimmunoassay which we recently described (Romani et al., 1980). The dependence of antibody binding on antigen concentration is presented in Figure 4. At 1:100 sera dilution, antigen saturation is reached in the presence of 50 μL of a solution of 50 μg of nucleosomal DNA per mL, i.e., with 2.5 μg of DNA. However, this test can easily detect 0.25 μg of chromatin DNA. Investigation of the dependence of antibody binding on antisera concentration indicated that specific reaction can be detected at sera dilutions varying from 1:10 to over 1:2000. Since γ-globulins tend to bind nonspecifically to chromatin, it is often difficult to use high sera concentrations with chromatin. In the radioimmunoassay the antigen–antibody complex is extensively washed, so that specific reaction can be detected at sera dilutions as low as 1:10. Binding of IgG to the antigen immobilized on the microtiter plate is fully inhibited by preincubation of the sera with nucleosomes. Under the experimental conditions presented in Figure 5, 1 μg of nucleosome inhibited the reaction by 33%. As pointed out elsewhere (Romani et al., 1980), the sensitivity of the reaction can be varied by altering the antigen

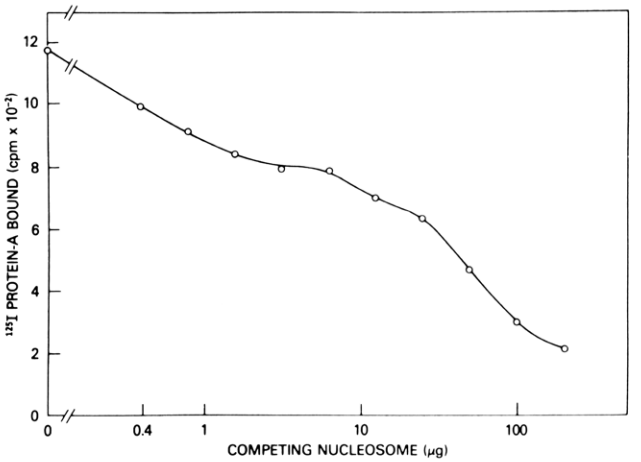


FIGURE 5: Inhibition of the binding of antinucleosome antibody to immobilized nucleosomes by free nucleosomes. Antiserum (IgG) at 1:50 dilution was incubated with an equal volume of 1% BSA in PBS containing increasing amounts of antigen. After centrifugation the unbound antiserum was added to wells precoated with 100 μL of 50 μg/mL chicken nucleosome.

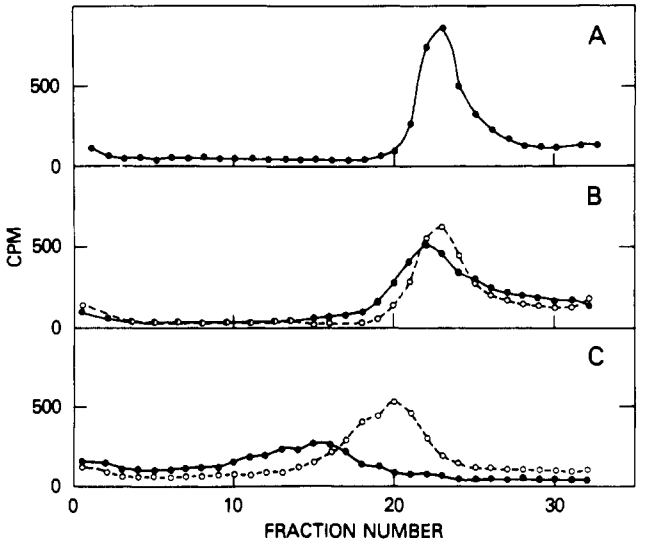


FIGURE 6: Antibody binding to 5′-$^{32}$P-labeled nucleosomes detected by immunosedimentation. 50 ng of 5′-$^{32}$P-labeled nucleosomes was incubated with antinucleosome serum and sedimented for 14 h in a 5–25.8% neutral sucrose gradient at 39 500 rpm in an SW40 rotor. (A) No antiserum; (B) 10.0 μg of IgG; (C) 100.0 μg of IgG. (●) IgG from antinucleosomes; (○) IgG from nonimmunized rabbits. Sedimentation was from right to left.

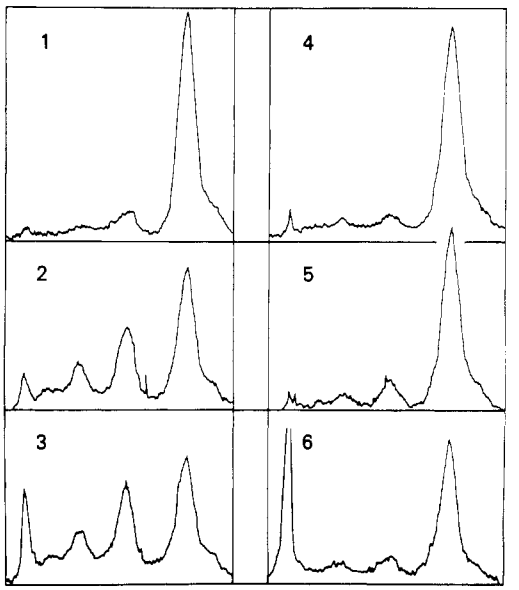


FIGURE 7: Antibody binding to 5′-$^{32}$P-labeled nucleosomes detected by electrophoresis in neutral polyacrylamide gels. 90 ng of nucleosome was incubated with increasing quantities of γ-globulins and then fractionated by using neutral polyacrylamide gels. The gels were exposed to X-ray film. Densitometer traces of the autoradiograph are shown. Channels 1–3, 1, 12, and 25 μg of antinucleosome per incubation; channels 4–6, 1, 12, and 25 μg of normal rabbit IgG. Migration was from left to right.

or the antisera concentrations and by increasing the specific activity of Protein A.

(*e*) *Immunosedimentation.* Purified nucleosomes were end labeled with polynucleotide kinase to give 5′-$^{32}$P-labeled chicken nucleosomes. The labeled nucleosomes were incubated with various amounts of either control normal rabbit IgG or IgG purified from antinucleosomes by DEAE chromatography. The resulting complexes were analyzed by centrifugation in neutral sucrose gradients. The data presented in Figure 6 indicate that binding of IgG molecules to nucleosomes could barely be detected when the weight ratio of IgG to nucleosome was 200:1 (Figure 6, panel B). At a weight ratio of 2000:1 the antinucleosome γ-globulin created a significantly higher molecular weight complex than the γ-globulins obtained from nonimmune sera. If it is arbitrarily assumed that the amount of nucleosome-specific γ-globulin constitutes 1% of the population of the γ-globulins in the immunized rabbits, then it can be calculated (using values for the molecular weight of a nucleosome as $3 \times 10^5$ and that of an IgG as $1.5 \times 10^5$) that a significant increase in the molecular weight of the nucleosome due to antibody binding requires a molar ratio of antibody to nucleosome of about 40. Similar studies utilizing affinity-purified antibodies to various histone fractions (Simpson & Bustin, 1976; Bustin et al., 1977b) indicated that a relatively large molar excess of antibodies over nucleosomes is required for demonstration of specific movement of nucleosomes in sucrose gradients.

(*f*) *Electrophoresis in Neutral Gel Systems.* Nucleosome–antibody complexes could also be detected by electrophoresis in nondenaturing polyacrylamide gels. 5′-$^{32}$P end-labeled nucleosomes were incubated with either control γ-globulins or γ-globulins purified from antinucleosome sera by chromatography on DEAE-cellulose. After incubation the mixtures were applied to 4% polyacrylamide gels. The location of the nucleosomes was visualized by autoradiography. Figure 7 presents scans of the autoradiograms which show that incubation with antinucleosome IgG brought about a significant and specific decrease in the mobility of the nucleosomes. The change in the mobility could be observed only at γ-globulin to nucleosome weight ratios of over 10 (not shown). When the weight ratio reaches 135 (panel 2, Figure 7), three peaks are clearly resolved. About 60% of the material migrates in the position of the unreacted nucleosome while the rest is distributed among two distinct, heavier peaks. A twofold increase in the weight ratio of γ-globulins to nucleosomes resulted in a relative decrease of the material migrating as the unreacted monomer with a concomitant increase in the slower moving material (panel 3). Further increase in the weight ratios results in a situation where the amount present as free monomer is smaller than that present in the slower migrating fraction (not shown). Note that addition of control γ-globulin did not result in production of discrete slower migrating radioactive peaks. Under the electrophoretic conditions used, γ-globulins do not migrate into the gel. Indeed, when the gels were stained with Coomassie blue, all the protein was localized at the origin of the gel. Such high protein concentrations often block the entrance of nucleosomes into the gel and radioactivity is accumulated at the origin (panel 6).

The data presented above indicate that it is possible to demonstrate specific reaction between nucleosomes and antinucleosomes by a variety of methods. Since nucleosomes are a complex macromolecular structure, it is possible that the antibodies are directed against only one component of the nucleosome. Therefore, efforts were made to identify the antigenic sites against which the antibodies are directed.

*Identification of Nucleosome Antigens Reacting with the Antinucleosome Sera.* (*a*) *Lack of Reaction with Isolated Components of the Nucleosome.* The antisera elicited by salt-washed nucleosomes which are devoid of detectable amounts of histone H5 (see Figure 1) contain significant amounts of antibodies specific to H5. This result suggests that the salt-washed preparations still contained small, undetectable amounts of H5 which elicited a strong immune response. The anti-H5 activity was removed by repeated passage of the γ-globulins on Sepharose 4B columns to which histone H5 was covalently bound. The antinucleosome IgG purified on anti-H5 columns still contained antibodies against salt-washed nucleosomes as measured by complement fixation (Figure 3),

Table I: Specificity of Antinucleosome Antisera[a]

| antigen | [$^{125}$I] Protein A bound (%) |
|---|---|
| salt-washed nucleosomes | 100 |
| DNA from nucleosomes (proteinase K, phenol, chloroform treated) | 3 |
| DNA from nucleosomes (3 M NaCl, hydroxylapatite) | 7 |
| calf thymus histones | 7 |
| DNA + histone H1 (1:1) | 4 |
| DNA + histone H2A (1:1) | 2 |
| DNA + histone H3 (1:1) | 1 |
| DNA + histone H4 (1:1) | 2 |
| chicken erythrocyte histones | 6 |
| cross-linked chicken erythrocyte histone octamer | 0 |
| cross-linked chicken core particles | 100 |
| calf thymus protein HMG-1 | 9 |
| calf thymus protein HMG-17 | 4 |
| micrococcal nuclease | 4 |
| actin | 0 |

[a] The various antigens were adsorbed to microtiter plates, and antibody binding was measured by solid-phase radioimmunoassay (Romani et al., 1980). The amount of antigen used was normalized to that present in nucleosomes. Binding is expressed as a percentage of the binding of antinucleosome to salt-washed nucleosomes on the same microtiter plates.

solid-phase radioimmunoassay (Figures 4 and 5), and indirect immunofluorescence (Figure 2). The data presented in Table I indicate that the antisera did not contain significant amounts of antibodies directed against the known major nucleosomal constituents. Thus, DNA purified from nucleosomes by treatment with proteinase K, phenol, and chloroform–isoamyl alcohol had only 3% of the activity present in the nucleosomes. DNA purified by 3 M NaCl and hydroxylapatite (Stein et al., 1977) had 7% of the activity. Unfractionated calf thymus histones, complexes of DNA extracted from chicken nucleosomes, individual calf thymus histone fractions, chicken histones, cross-linked histone octamer from chicken nucleosomes (Stein et al., 1977), and nonhistone proteins HMG-1 and HMG-17 did not exhibit significant antigenic activity toward antinucleosome when measured by solid-phase radioimmunoassay. Similarly, the antisera did not contain antibodies to the micrococcal nuclease which was used to prepare the nucleosomes and which may remain associated with the purified monomer (Levinger & Carter, 1979). Microcomplement fixation tests were in full agreement with the results obtained by solid-phase radioimmunoassay.

(*b*) *Nuclease and Protease Sensitivity.* The data presented in panels A and B of Figure 8 reveal that antibody binding to nucleosome is dependent on the structural integrity of the proteins present in the complex. Thus, a 5-min digestion of nucleosomes with proteinase K at a substrate/enzyme ratio of approximately 100 brought about a loss of 70% of antibody binding (panel B). All reactivity was lost by a 15-min digestion at a substrate/enzyme ratio of 3. A similar effect was noted with trypsin. A 5-min digestion of a substrate/enzyme ratio of 800 brought about a 50% reduction of antibody binding. All antibody binding was lost at a substrate/enzyme ratio of 500 (panel A). Electrophoresis of the particles after proteinase digestion revealed that the histones were degraded (not shown). We conclude that loss of antigenicity is linked to digestion of histones.

The antibody binding was not susceptible to redigestion of the particles with micrococcal nuclease (not shown). Digestion with deoxyribonuclease 1 (panel C) reduced the binding by 60%. A 10-fold increase in enzyme/substrate ratio did not reduce further the amount of antibody bound. Thus, part of

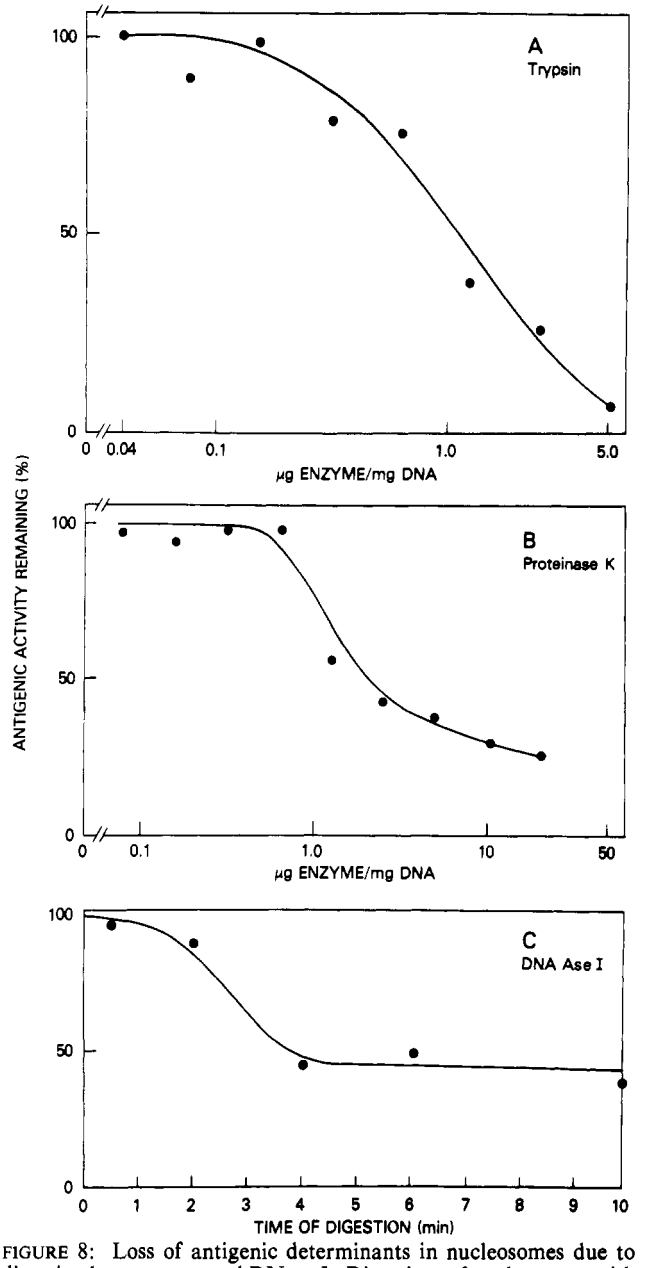


FIGURE 8: Loss of antigenic determinants in nucleosomes due to digestion by proteases and DNase I. Digestions of nucleosomes with serial dilutions of (A) trypsin and (B) proteinase K. 5 μg of nucleosome in a total volume of 100 μL was incubated with serial dilutions of protease in 10 mM Tris-HCl and 1 mM EDTA, pH 7.5, for 5 min at 30 °C. Reactions were terminated by addition of 2 mM phenylmethanesulfonyl fluoride (PMSF). (C) Time course of DNase I digestion. 42 μg/mL nucleosome was incubated with 60 units of DNase I in 10 mM Tris-HCl, 5 mM $MgCl_2$, and 1 mM PMSF, pH 7.5, at 37 °C. 100 μL samples were removed at intervals, added to microtiter plates containing 11 μL of 200 mM EDTA, pH 7.2, and tested for antigenicity.

the antibodies in the serum can bind to the chromatin particles even though the DNA is no longer intact.

It has been reported that in DNase I digested nucleosomes the DNA remains associated with the histone octamer to give a "nicked" 11S particle (Noll, 1977). This is not the case with protease treatment where the N-terminal regions of the histone separate from the main body of the nucleosome after digestion (Simpson & Whitlock, 1977). Since all the antigenic activity was lost upon protease digestion and at least 50% of the activity was lost upon nuclease digestion, we conclude that at least 50% of the antibody species present in the serum are directed against antigenic determinants, which are nucleoproteins.

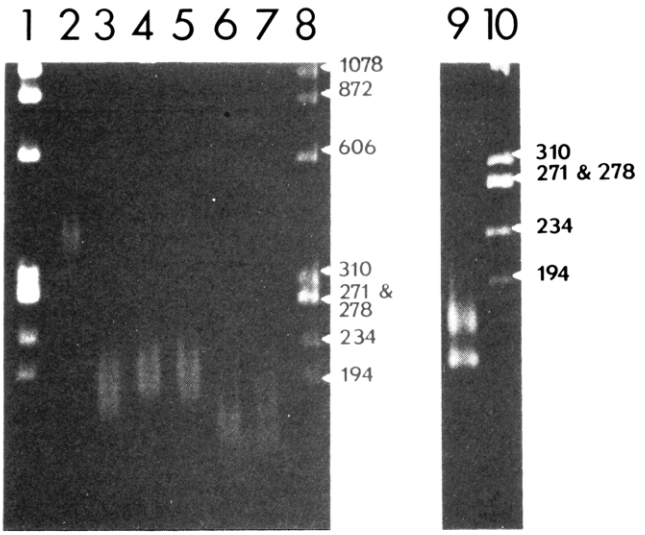


FIGURE 9: DNA size of chromatin particles tested for antinucleosome activity. The location of the DNA was visualized by ethidium bromide. From left to right the channels contain (1) $\phi$X174 *Hae*III standard, (2) DNA from chicken dinucleosomes, (3) DNA from HeLa nucleosomes, (4) DNA from chicken nucleosomes, (5) same as (4), (6) DNA from wheat germ nucleosome, (7) DNA from rat liver nucleosome, (8) same as (1), (9) DNA from chicken core particle, and (10) same as (1).

*Independence on Chromatin Repeat Size and Dependence on Species Specificity.* To determine whether the antiserum recognizes features exclusively characteristic to the monomer nucleosomes, we have reacted chromatin stretches containing various numbers of nucleosomes with the antinucleosome sera. To determine whether the antiserum recognizes features which are universal to nucleosomes extracted from various sources, we isolated chromatin particles from different tissues and tested with the antiserum.

Figure 9 presents the size and purity of the DNA from chicken core particles, from the monomers obtained from the four tissues studied, and from the dimers obtained from chicken. It can be seen that in all cases the antigens tested were reasonably pure.

The extent of reaction with chromatin of various repeat sizes obtained from different sources is presented in Table II. With chromatin particles obtained from chicken, the core particle (145 base pair DNA), nucleosome (core particle + linker DNA, 195 base pair DNA), dimer, and trimer react equally well. We conclude that the antibodies are not directed against the linker DNA between two nucleosomes and that the antibodies do not recognize features exclusively specific to the intact mononucleosome. Subnucleosomal particles (sedimenting somewhat slower than the 11S particle in a sucrose gradient and displaying a heterogeneous array of DNA fragments smaller than 120 base pairs of DNA) react as well as nucleosomes, suggesting that most of the antibodies recognize determinants composed of histones and small segments of DNA. Thus, the antibodies may be regarded as directed against determinants whose binding requires intact DNA–protein contacts but is not dependent on the integrity of the entire nucleosome. Data presented in Table II also indicate that the antibodies recognize differences between chromatin particles obtained from various sources. We conclude, therefore, that nucleosomes do not contain specific serologic features which would elicit antibodies directed against all chromatin particles, regardless of their origin. The specificity we found probably reflects the limited species specificity of histones. The species specificity of histones H2A and H2B is known to be enhanced in the N-terminal portion of the

Table II: Binding of Antinucleosome Serum to Homologous and Heterologous Subnucleosomes and Mono-, Di-, and Trinucleosomes[a]

| | [$^{125}$I] Protein A bound (%) | | | | |
|---|---|---|---|---|---|
| tissue | subnucleosome (<145-bp[b] DNA) | core (145-bp DNA) | monomer | dimer | trimer |
| chick erythrocyte | 82 | 100 | 100 | 100 | 85 |
| HeLa | nt[c] | nt | 83 | nt | nt |
| rat liver | 8 | nt | 9 | 9 | 5 |
| wheat germ | 7 | nt | 2 | 0 | nt |

[a] Binding was determined by solid-phase radioimmunoassay and is expressed as a percentage of homologous binding. [b] bp = base pairs. [c] nt = not tested.

molecules (Von Holt et al., 1979) which bind to DNA.

We have also considered a possibility that the antibodies are elicited against a tissue-specific nonhistone protein which, like histone H5, is found only in very small amounts but is very immunogenic. The existence of such an antigen has been suggested in a preliminary report (Einck & Woodcock, 1978). The proteins present in chicken nucleosomes or in chick erythrocyte whole chromatin have been fractionated by electrophoresis in a 20% polyacrylamide gel cross-linked with 1% *N,N'*-diallyltartardiamide (DATD) and run in the presence of 0.1% sodium dodecyl sulfate to check for such a situation. The gel was relaxed, and the proteins were tranferred and covalently bound to diazobenzyloxymethyl (DBM) paper. The paper was reacted with antinucleosome followed by [$^{125}$I]-Protein A (Renart et al., 1979). Control studies using antisera to histones or to HMG-1 chromosomal proteins indicate that it is possible to use this technique to pinpoint the location of a chromosomal antigen in the gel. These data are presented in Figure 10. The Coomassie blue stain of the transferred gel indicates the location of chromosomal proteins of the chicken erythrocyte (panel A, column 2) and of the H5 (column 1) markers. The autoradiogram after treatment with antinucleosome (panel B) indicates that only histone H5 gave a significantly positive reaction. The lack of reaction with the core histones or other putative antigens in the preparation is not due to insufficient transfer. All the histones and some of the HMG proteins can be specifically stained by this procedure. An example is shown in panels C and D of Figure 10. Panel C indicates the location of histones isolated from purified chicken nucleosome (lane 3), of the proteins present in a 5% perchloric acid extract of chicken chromatin (lane 4), and of the proteins present in wheat germ mononucleosomes which have been extracted with 0.6 M NaCl (lane 5). The autoradiogram of the corresponding DBM paper after treatment with antisera specific to histone H4 (panel D) clearly shows that only histone H4 bound antibodies. After removal of bound anti-H4 by urea treatment (Renart et al., 1979), the paper was treated with antinucleosome sera which had been passed through the Sepharose H5 column. The data presented in panel E clearly show that the antibodies present in the sera do not react with any component present in purified chicken mononucleosomes. The lack of reaction with H5 attests to the fact that anti-H5 antibodies have been effectively removed from the sera. When the paper was retreated with anti-H4 or anti-H3 sera, the corresponding proteins were visualized (data not shown). This indicates that the proteins were covalently linked to the DBM paper and the urea treatment used to remove the first antibodies did not remove the antigens from the paper. These results minimize the possibility that some

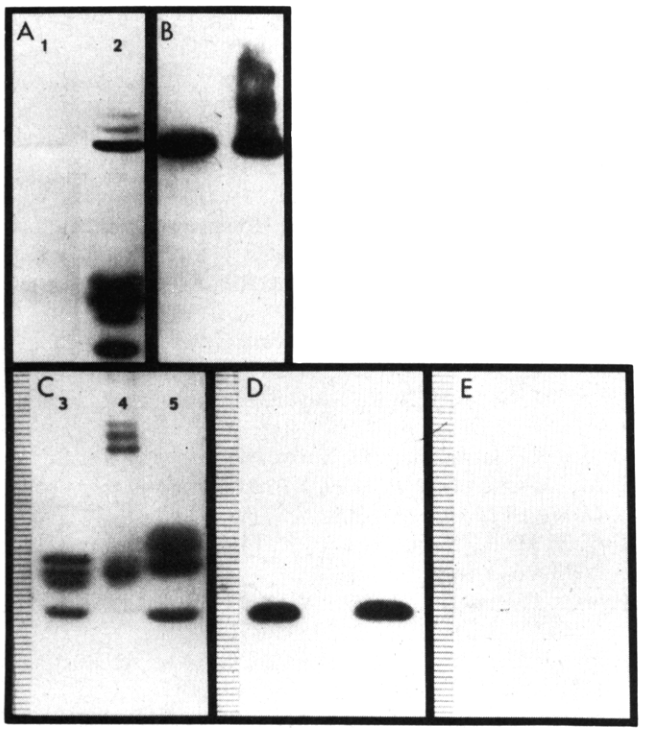


FIGURE 10: Search for antigens in chromosomal proteins. Chromosomal proteins from chicken erythrocytes and marker proteins were electrophoresed on 20% polyacrylamide gel. After the gel was relaxed, the proteins were transferred to diazobenzyloxymethyl paper. The location of the antigens was visualized by autoradiography (see Materials and Methods for details). Panels A and C depict polyacrylamide gel slabs which were stained with Coomassie blue after partial transfer of the proteins in DBM paper. Lane 1, histone H5; lane 2, chicken erythrocyte chromosomal proteins; lane 3, proteins present in purified chicken erythrocyte chromatin particles (immunogen); lane 4, proteins present in a 5% perchloric acid extract of chicken erythrocyte chromatin; lane 5, proteins present in purified wheat germ chromatin particles. (Panel B) Autoradiogram of the DBM paper corresponding to panel A after treatment with crude antinucleosome sera. (Panel D) Autoradiogram of the DBM paper corresponding to panel C after treatment with anti-histone H4 serum. (Panel E) Autoradiograph of panel C after treatment with antinucleosome serum from which anti-H5 activity was removed by affinity chromatography.

unidentifiable nonhistone protein elicited significant amounts of antibodies and provide further support for the conclusion that the nucleosome elicited antibodies directed against nucleoprotein complexes in the chromatin particle.

## Discussion

The results presented lead to the conclusion that most of the antibodies elicited by chicken erythrocyte nucleosomes are specific toward nucleoprotein determinants present in the chromatin particle. The binding of antibodies to the nucleosome is dependent on the presence of both protein and DNA but is independent of the structural integrity of the entire nucleosome.

The presence of specific antibodies in antisera elicited by nucleosomes can be detected by passive immunodiffusion, microcomplement fixation, immunofluorescence, solid-phase radioimmunoassay, electrophoresis of 5′-$^{32}$P-labeled particles in neutral polyacrylamide gels, and immunosedimentation. Since in immunosedimentation experiments all the nucleosomes bound antibodies and sedimented as a heavier complex than nucleosomes reacted with control $\gamma$-globulins, it can be concluded that the antibodies are not produced by a special subset of nucleosomes.

It is important to note that the salt-washed particles, which by accepted electrophoretic techniques contain only the four core histones (see Figure 1), elicited antibodies against the erythrocyte-specific H5. It is known that histone H5 is significantly more immunogenic than the conserved core histones [for review, see Bustin (1978)]. This is an example where a small amount of undetectable contaminant is highly immunogenic and, therefore, a relatively large proportion of the antibodies present in the antisera is directed against this contaminant. We have already pointed out that, when possible, it is advantageous to use antisera elicited by a chromosomal protein purified from one source to study the organization of its homologue in another tissue (Bustin et al., 1977a).

None of the known constituents of the nucleosome elicited detectable amounts of antibodies. Thus, the antinucleosome sera did not react with DNA, cross-linked histone octamer, individual histone fractions, or complexes of DNA and individual histone fractions. These findings are in agreement with previous studies which indicated that native DNA is not immunogenic (Stollar, 1973) and that histones in their native, chromatin-bound, state do not elicit significant amounts of antibodies (Goldblatt & Bustin, 1975). Furthermore, in a recent short report, Einck & Woodcock (1978) presented similar findings. Hannestad & Stollar (1978) noted that certain rheumatoid factors react with nucleosomes. These naturally occurring human autoantibodies seem to have an immunological specificity similar to that of the experimentally induced sera described in this manuscript (Rekvig & Hannestad, 1979).

Taken at face value, these results can be interpreted as indicating that the antibodies are directed against a set of antigenic sites present on the intact nucleosome. The finding that the core particle (145 base pair DNA) and the nucleosome dimer and trimer react to the same degree suggests that the antibody is not directed toward the linker region. Since the size of the core particle is conserved, it was expected that chromatin particles obtained from various sources would cross-react. The data presented in Table II clearly indicate that this is not the case. The fact that the submonomer fraction which is known to contain histone aggregates complexed with DNA of various chain lengths (Weintraub, 1975; Nelson et al., 1977; Bakayev et al., 1977) reacts also indicated that the entire, intact, structure of the nucleosome is not required for antigenic activity. We have also noted that exposure of the nucleosome to urea denaturation does not abolish the activity (unpublished results). Apparently certain nucleoprotein antigenic sites survive perturbation in nucleosome structure. Protease digestion fully abolished the immunological activity, suggesting an obligatory protein component. DNase I digestion reduced the activity by over 50%, suggesting that at least part of the antibodies also require DNA for reaction. Since it is known that in DNase I digested nucleosomes part of the DNA remains attached to the histone octamer (Noll, 1977), it seems safe to assume that at least part of the remaining antigenic determinants are also nucleoproteins.

We conclude that the antigenic sites on the surface of the nucleosome consist of a DNA segment complexed with part of the N-terminal portion of the histones. Such a situation can explain the total loss of antigenicity even after mild trypsin digestion; the N-terminal region of the histones is known to be rapidly digested (Weintraub & Van Lente, 1974) and does not stay associated with the main body of the nucleosome (Simpson & Whitlock, 1977). Variability in the primary structure of the histones is enhanced in the N-terminal region, a fact which could account for the species specificity of the immunological reaction. Obviously, it can be expected that

most of the antibodies will be elicited against the histone regions which are specific to erythrocyte histones and not toward the invariant histone regions which are shared among all species. The lack of reaction with nucleosomes isolated from wheat germ could be explained by the fact that the N terminals of the histones present in this organism are significantly different from those belonging to the chicken. The lack of reaction with the rat nucleosomes can be explained by the fact that the rat is phylogenetically related to the rabbit, the animal used to elicit antibodies.

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