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Monoclonal Antibodies against Distinct Determinants of Histone H5 Bind to Chromatin†

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ABSTRACT: A series of monoclonal antibodies specific for distinguishable epitopes in chromosomal protein histone H5 were obtained from mice immunized with either free H5 or H5·RNA complexes. The antibodies elicited by H5 could be distinguished from those elicited by H5·RNA by their binding to native or acid-denatured H5, by their interaction with the globular region of H5, and by their cross-reactivity with H1°. The specificity of the antibodies was assessed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting experiments. The antibodies could distinguish between H5 and the closely related histones H1 and H1°. The binding of some of the antibodies to the antigens was dependent on the type of assay used, suggesting nonrandom binding of the antigen to the solid supports used in ELISA and immunoblotting. Competitive ELISA experiments indicate that 8 of the 11 antibodies characterized bind to distinct epitopes. Three monoclonal antibodies bind to epitopes which are in close spatial proximity, causing mutual steric hindrance. The monoclonal antibodies bind to nuclei of fixed cells and to isolated chromatin, indicating that the epitopes are present both in the purified protein and in chromatin-complexed H5. These monoclonal antibodies can be used to study the organization of distinct regions of histones H5 and H1° in chromatin and chromosomes.

The lysine-rich histone group differs from the core histones in its tissue and species specificity and in the manner in which it is organized in chromatin. While the core histones are highly conserved during evolution (Elgin & Weintraub, 1975), the lysine-rich histones display a remarkable species and tissue specificity (Bustin & Cole, 1968). The core histones are organized into an octamer around which the nucleosomal DNA is wound, while the lysine-rich histones are not an integral part of the core particle (Kornberg, 1977; McGhee & Felsenfeld, 1980). Several types of experiments suggest that the lysine-rich histones stabilize the structure of the nucleosomes and are involved in the maintenance and control of higher order chromatin structure (Simpson, 1978; Finch & Klug, 1976; Bradburg et al., 1973; Renz et al., 1977). The various functions of the very lysine-rich histones seem to be dependent on specific structural features of the molecule. It has been shown that these histone molecules are composed of three main structural domains (Bustin & Cole, 1970; Chapman et al., 1977) which exert specific effects at various levels of chromatin organization (Allan et al., 1980; Thoma et al., 1983). In most cells, histone H1 is the major very lysine-rich histone species (Elgin & Weintraub, 1975). However, in the nucleated erythrocytes of several species, the H1 molecules are largely replaced by the H5 lysine-rich histone variant (Neelin et al., 1964). Recent studies indicated that most eukaryotic cells have a minor very lysine-rich histone variant, histone H1°, which is closely related to histone H5 (Panyim & Chalkley, 1969; Pehrson & Cole, 1981; Smith et al., 1980). The H1° molecule seems to accumulate in cells which either are terminally differentiated or have a lower rate of cell division (Pehrson & Cole, 1980; Keppel et al., 1977; Chabanas, 1983). The molecular basis of this observation is not understood; however, it is known that H5 imparts greater stability to higher order chromatin structure than does H1 (Thomas & Rees, 1983; Kumar & Walker, 1980). The H1/H5 histones bind to chromatin through a globular domain which seems to locate the molecule at the exit and entrance points of the 165 base pair DNA in the chromatosome (Simpson, 1978). Sequence analysis (Pehrson & Cole, 1981; Smith et al., 1980) and immunological studies have shown that the central globular region of H1 differs from that of H5 and H1° (Mura & Stollar, 1981; Allan et al., 1982). Thus, it is possible that the functional differences between H5/H1° and H1 are related to structural features in the globular region of these proteins.

Immunological approaches have been used for various studies on the specificity of histones and on their role in maintaining the structure and regulating the function of chromatin (Bustin, 1979). So far, most of the serological studies on chromatin were done with polyclonal sera against specific proteins or histone peptide (Bustin, 1979; Absalom & Regenmortel, 1978; Muller et al., 1983; Mathiu et al., 1981). Defined monoclonal antibodies may be useful in studying the organization of specific histone regions in chromatin. Such studies may be particularly applicable to the lysine-rich group since we have shown that in chromatin the antigenic determinants in this histone are relatively exposed (Goldblatt & Bustin, 1975). Defined monoclonal antibodies may provide further insight into the organization of particular regions of the molecules in chromatin and chromosomes.

In the present paper, we describe the preparation and characterization of several monoclonal antibodies against histone H5. The antibodies can be used to distinguish between

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H1, H1°, and H5 and bind to their antigenic determinants in fixed cells and in purified chromatin. Evidence is presented that most of the antibodies bind to a distinct epitope. The location of the epitope is mapped in further detail in the following paper (Mendelson et al., 1984).

Materials and Methods

Histone H1 was prepared as previously described (Goldblatt & Bustin, 1975). Ox liver histone H1° was a gift from Dr. B. Smith, Chester Beatty Institute, England. Chicken histone H5 and the globular region of H5 were obtained from Dr. J. Allan (Allan et al., 1980).

Polyclonal antibodies to H5 were obtained by immunizing rabbits with nucleosomes purified from chicken erythrocytes. The specificity of the sera was tested by enzyme-linked immunosorbent assay (ELISA)[1] and by immunoblotting on DBM paper (C. S. M. Tahourdin and M. Bustin, unpublished results).

Monoclonal Antibodies. Female BALB/c mice were immunized either with pure H5 or with H5·tRNA complexes (3:1 w/w). Routinely, the mice were immunized with 15 μg of H5 emulsified in 200 μL of 66% complete Freund's adjuvant injected intraperitoneally, followed by weekly boosts of H5 in incomplete Freund's adjuvant and in phosphate-buffered saline. After three weekly boosts, the mice received an intravenous boost which was administered either 5 or 11 weeks after the first immunization. Three days after the intravenous injection, the mice were bled from the eye, and spleens from the mice which were positive for anti-H5 acitivity were removed and used for hybridoma production. For fusion, 1.0×10^8 spleen cells were mixed with about 2.4×10^7 myeloma P_3X63-AG8-U1 or $SP_2/0$ cells in 30% PEG-1000 (Baker). After fusion, the cells were seeded in 96-well microtiter plates. The supernatants were tested for antibody production by an ELISA assay (Engvall & Perlman, 1972; Bustin et al., 1982) using H5 as antigen. Hybrid cells from positive wells were subcloned 2 times at limiting dilution in media containing 20% culture supernatant from the parental cell line.

Purification of Antibodies from Culture Supernatants. Hybrids in growth phase were grown in serum-free medium HB101 (Hana Biologicals). Supernatants from these cultures were concentrated 10–20-fold by ultrafiltration with a PM30 Amicon membrane. Some of the antibodies were purified by passage through a protein A–Sepharose column (Pharmacia) at a ratio of 300 mL of culture supernatant to 5 mL of resin. Bound immunoglobulins (Ig's) were eluted with 3 M guanidine hydrochloride, pH 4.5. The purified antibodies gave the same results as the concentrated supernatants.

Immunoassay and Immunofluorescence Procedures. Solid-phase radioimmune assay using ^{125}I-labeled sheep anti-mouse Ig (NEN) and enzyme-linked immune assay were performed as described previously (Romani et al., 1980). Usually the antigen was added to the plates in 100 μL of sodium phosphate, pH 7.0, and the plates were incubated for 16 h at 4 °C. The unbound antigen was washed with PBS–Tween, the wells were blocked with 1% horse serum in PBS, and the antibody was diluted in 1% horse serum in PBS added for 2–3 h at room temperature. After removal of unbound antibody, the second antibody was added for 2 h followed by substrate additions. The A_{405} that developed was read automatically in a multiscan ELISA reader (Flow).

Ouchterlony immunodiffusion was performed at pH 8.0 in plates obtained from Cappel Laboratories. The type of Ig produced was determined with anti-Ig sera obtained from Miles Laboratories.

Indirect immunofluorescence was performed with chicken erythrocytes, air-dried on microscope slides, or with KD human fibroblasts grown in Lab Tech tissue culture chambers. The cells were fixed and permeabilized with methanol and acetone at −20 °C. The staining procedure was done as previously described (Bustin & Neihart, 1979) using undiluted culture supernatants and fluorescein-labeled goat anti-mouse Ig obtained from Cappel Laboratories. The slides were observed and photographed with a Zeiss III photomicroscope.

Electrophoresis in Polyacrylamide Gels and Immunoblotting. Proteins were electrophoresed in either 12% or 18% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Laemmli, 1970). Transfer of proteins from polyacrylamide gels to nitrocellulose was done electrophoretically essentially according to Lin & Kasamatsu (1983). The proteins were transferred to 0.15-μm nitrocellulose membranes (Schleicher & Schuell) at 500 mÅ for 18 h at 15 °C in 25 mM Tris-HCl, pH 8.3, 190 mM glycine, and 20% methanol. Nonspecific binding to nitrocellulose was prevented by incubation in 3% bovine serum albumin in 50 mM Tris-HCl, pH 7, 0.15 M NaCl, and 0.1% NaN_3 for 1 h at 37 °C. The blocked nitrocellulose membranes were incubated in a 1:10 dilution of monoclonal supernatants or in 1:200 mouse serum in the same buffer for 12–24 h at room temperature with constant shaking. Unbound antibodies were removed with three washes of 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.1% NaN_3 at room temperature, and the antibodies bound to nitrocellulose were detected with ^{125}I-labeled sheep anti-mouse Ig (NEN) added at an activity of 2×10^6 cpm/mL. After 1-h incubation, with constant shaking, at room temperature, the nitrocellulose sheets were washed 3 times with the above buffer, and the dried sheets autoradiographed.

Chromatin Isolation. Chromatin was isolated from washed chicken erythrocytes. The cells were lysed in 0.2% Triton, 100 mM KCl, 50 mM Tris, pH 8, 1 mM $MgCl_2$, and 0.1 mM PMSF and the nuclei purified by centrifugation through 2.2 M sucrose in the same buffer. The pellet was washed in 0.25 mM EDTA, pH 7.5, and 0.1 mM PMSF, and the nuclei were broken by homogenization. The viscous pellet was dialyzed and an aliquot briefly digested with micrococcal nuclease (McGhee et al., 1980).

Results

Antibody Production and Characterization. Female BALB/c mice were immunized with either H5 or H5·RNA complexes as described under Materials and Methods. Several fusions resulted in 770 hybrids which were tested for production of antibodies specific to histone H5. From a total of 20 positive hybrids, 11 were successfully cloned and stabilized in tissue culture. After the clones stabilized, the cells were grown in serum-free media, and the supernatants from these cultures were concentrated and used to determine the type of antibody produced by each hybrid cell line. Ouchterlony immunodiffusion (Figure 1) revealed that each culture secreted only one type of Ig and that the various clones secreted different types of Ig. Thus, from the 11 supernatants, 4 belonged to the IgM class, 3 to IgG_1, 1 to IgG_{2a}, 2 to IgG_{2b}, and 1 to IgG_3. We conclude that the immune response to histone H5 is not restricted to a single class of Ig.

The binding of the antibodies to histone H5 was dependent on the concentration of both the antiserum and the antigen.

[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; DBM, diazobenzyloxymethyl; Ig, immunoglobulin; PBS, phosphate-buffered saline; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

Table I: Specificity of Monoclonal Antibodies to H5

antigen	absorbance at 405 nm[a] with antibody											
	1C3	6E12	4C6	3H9	3E8	2C8	1G11	2E5	2E12	2H5	4H7	mouse serum
H5	0.55	1.15	1.15	0.91	0.65	0.56	0.58	0.60	0.4	0.72	0.50	>2.0
GH5	1.6	0.84	1.25	1.10	1.18	1.10	0.0	0.0	0.06	0.0	0.0	>2.0
H5/GH5	0.34	1.37	0.95	0.82	0.55	0.57	∞	∞	6.6	∞	∞	1.0
H1°	0.76	0.04	0.07	1.1	0.02	0.43	0.0	0.0	0.04	0.01	0.03	1.45
H1	0.0	0.0	0.0	0.0	0.1	0.0	0.15	0.1	0.0	0.0	0.25	0

[a] Antigens were bound to the microtiter plates at a concentration of 5 μg/mL overnight, at 4 °C. Antibodies were used as serum-free culture supernatants. Mouse serum was diluted 1:200. Optical density was measured 1 h after the addition of substrate.

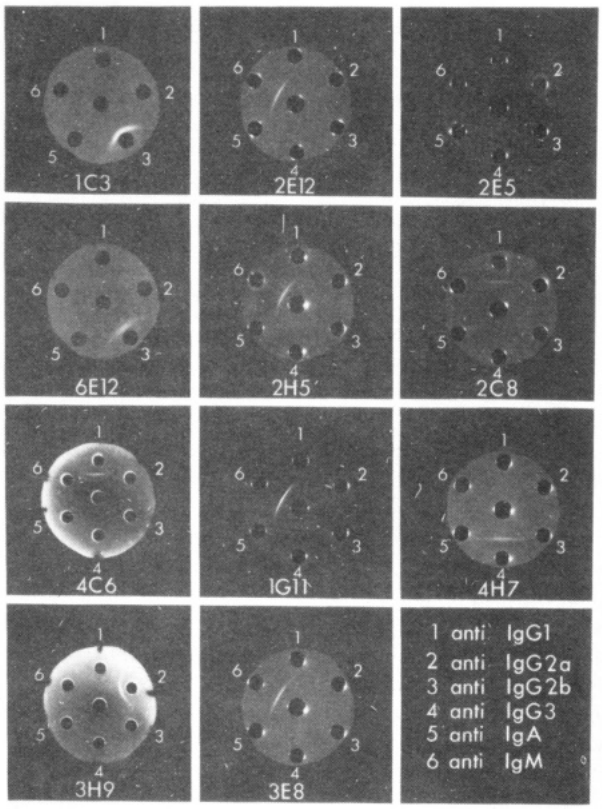

FIGURE 1: Ouchterlony double immunodiffusion on serum-free culture supernatants. The center well in each plate contained 10× concentrated media. The content of the peripheral wells is diagrammed in the lower right-hand panel.

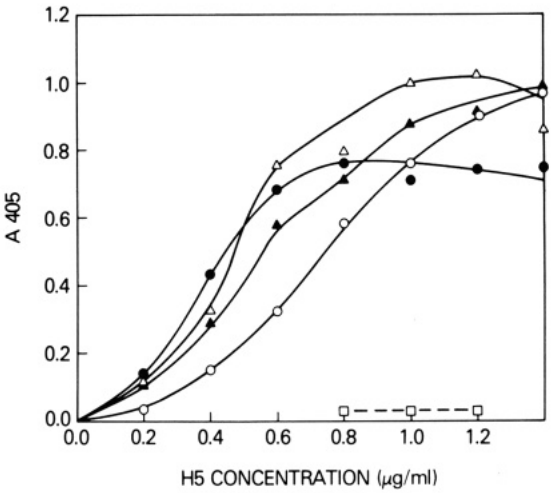

FIGURE 2: Enzyme-linked immunosorbent assay demonstrating the dependence of antibody binding on antigen concentration. Monoclonal antibodies 1C3 (●), 6E12 (○), 4C6 (▲), and 3H9 (△) were reacted with various concentrations of H5 for 2–5 h. The optical density was read 1 h after the addition of the substrate. (□) Reaction with control monoclonal antibody.

In the absence of antigen, none of the antisera bound to the plates, and, as expected, H5 did not give a positive reaction with several control monoclonal antibodies. An enzyme-linked immunoassay demonstrating the dependence of substrate hydrolysis (measured as the absorbance at 405 nm) on the concentration of the antigen is presented in Figure 2. Under standard assay conditions (see Materials and Methods), undiluted culture supernatants detected H5 in the same concentration range as a 5000-fold dilution of rabbit antiserum (Allan et al., 1982). The monoclonal antibodies could reliably detect less than 10 ng of H5 per microtiter well. (Routinely, the antigen was added to the well in 50–100 μL of solution of 200 ng/mL.)

Serological Specificity. Histone H5 is a highly specialized variant of the lysine-rich histone group. Polyclonal antibodies to H5 cross-react with yet another specialized H1 variant, histone H1° (Mura & Stollar, 1981; Allan et al., 1982). Since H5 and H1° are immunologically related and H1° has common sequences with H1, a monoclonal antibody could potentially either recognize all or distinguish among these three proteins. To establish the specificity of the various monoclonal antibodies, each antibody was reacted with histone H5 and the globular region of histone H5, GH5, as well as with histones H1° and H1 purified from calf thymus. Representative results demonstrating the reaction of monoclonal antibody 4C6 and 1C3 with the various antigens at several sera dilutions are presented in Figure 3. It can be seen that the two monoclonals displayed different specificity toward the various antigens. Monoclonal 4C6 and 1C3 with the various antigens at several sera dilutions are presented in Figure 3. Monoclonal 4C6 reacted with both GH5 and H5 and did not bind to H1° or to H1 while antibody 1C3 displayed a very strong reaction with GH5 and a somewhat weaker affinity for either H1° or H5. Thus, antibody 4C6 could be used to distinguish between proteins H5 and H1°. The difference in the specificity between the two antibodies suggests that the antibodies recognize different epitopes on the H5 molecule. Table I summarizes the specificity of all the monoclonal antibodies used in this study. Examination of the ELISA data indicates that, with respect to the relative binding to H5 and GH5, the monoclonal antibodies can be divided into three main categories. Monoclonals 1C3, 3E8, and 2C8 display a definite preference for binding to GH5 while clones 1G11, 2E5, 2E12, 2H5, and 4H7 secrete antibodies that react significantly better with the intact H5 than with the GH5 region. Polyclonal mouse serum and monoclonals 6E12, 4C6, and 3H9 have equal reactivity with both H5 and GH5. the antibodies could also be categorized with respect to their cross-reactivity with H1° Monoclonals 3H9, 1C3, and 2C8 and the polyclonal antibodies reacted strongly with H1°. These antibodies do not distinguish between H1° and H5, suggesting that they are directed against homologous regions in the two proteins. Monoclonals 6E12, 4C6, 3E8, 2E12, 2H5, and 4H7 displayed a weak reactivity with H1°. Most probably, these antibodies are directed against epitopes of partial homology between H1° and H5. Antibodies

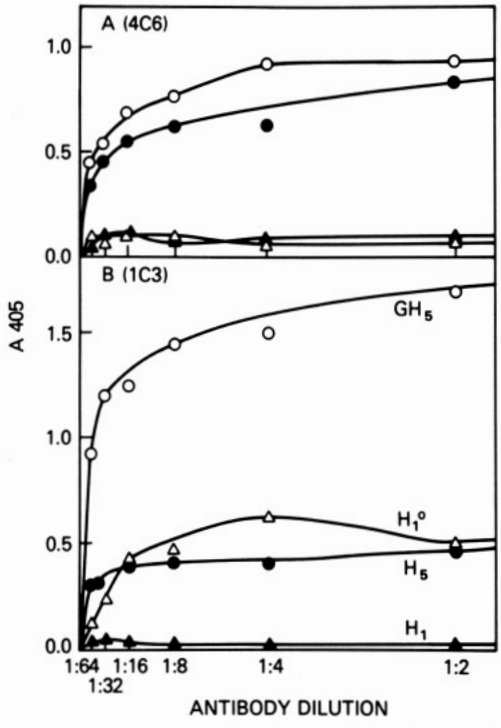

FIGURE 3: ELISA demonstrating the specificity of monoclonal antibodies 4C6 (A) and 1C3 (B) against various lysine-rich histones: (○) globular region of H5; (●) H5; (▲) calf thymus H1; (△) ox liver H1°. The antigen concentration added to the plate was 5 μg/mL. Absorbance was measured 2 h after addition of substrate.

1G11 and 2E5 did not react with H1°, indicating that they are directed against epitopes present in H5 but absent from H1°. Interestingly, the same antibodies failed to recognize the GH5 region. It has been already demonstrated that the immunological cross-reactivity between H5 and H1° is mostly due to antibodies directed against the globular region of the two proteins (Allan et al., 1982). Thus, these two antibodies may recognize epitopes located in the periphery of the globular region or in the nonglobular region of the molecule. Monoclonals 1G11, 2E5, 4H7, and 3E8 recognized epitopes present in calf thymus H1. The first three antibodies did not react with GH5. Monoclonals 1G11 and 2E5 may be directed against epitopes present in H5 and H1 but absent from H1°.

The specificity of the various monoclonals was further verified by immunoblotting on nitrocellulose paper. H5, GH5, H1, and H1° run on 18% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate were transferred to nitrocellulose paper, and the paper was incubated with various monoclonal antibodies diluted 1:10. The bound Ig's were detected with ^{125}I-labeled sheep anti-mouse Ig. Representative results are shown in Figure 4. In agreement with the data shown in Table I, antibody 3H9 reacted to the same degree with H5, H1°, and GH5; 1C3 displayed a stronger binding to GH5 than to H5 or H1°, and 4C6 displayed a significant preference for H5 over H1°. For quantitation, the autoradiograms were scanned with a Beckman DU-8 gel scanner. Integration of the areas under the H5 and GH5 peaks for 1C3 gave an H5/GH5 ratio of 0.75. Interestingly, antibody 6E12, which in the ELISA assay reacted with GH5, did not bind to GH5 on the nitrocellulose paper. We have verified that the GH5 molecule indeed transferred from the gel to the nitrocellulose paper by treating with nitrocellulose sheet with monoclonal 1C3. After this treatment, the GH5 molecule gave a strong autoradiographic spot, indicating that the GH5 molecule is indeed bound to the nitrocellulose paper. The converse situation, where monoclonal antibodies which do not

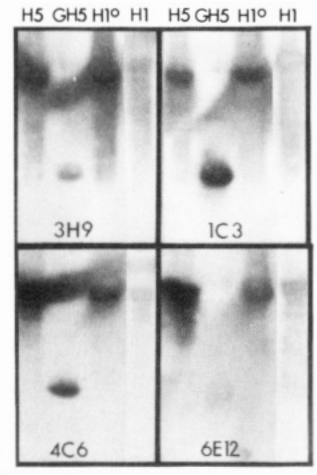

FIGURE 4: Immunoblots demonstrating the specificity of monoclonal antibodies 3H9, 1C3, 6E12, and 4C6 for H5, GH5, H1, and H1°. Ten micrograms of each protein was electrophoresed in an 18% polyacrylamide gel, transferred to nitrocellulose, and reacted with monoclonal antibody diluted 1:10 and with ^{125}I-labeled sheep anti-mouse Ig.

recognize GH5 in the ELISA test give a strong reaction on the nitrocellulose transfer test, was observed with monoclonals 4H7, 2E5, and 2H5 (not shown). The results suggest that the antigen binds to the solid supports in a nonrandom fashion. Alternatively, each assay may selectively destroy or denature particular regions in the molecule.

Effect of Denaturation on Antibody Binding. To gain insight into the nature of the antigenic determinants, the antigen was bound to the wells of the microtiter plates in 0.1% acetic acid, pH 2.5. The interaction of each monoclonal antibody with the H5 immobilized on the plates under acidic, denaturing conditions was compared to the binding obtained when the antigen was immobilized in phosphate buffer, pH 7.0. The results presented in Figure 5 indicate that the various antibodies bound to the denatured antigen in three distinct patterns. Polyclonal serum and monoclonals 2E5 and 4H7 bound equally well regardless of whether the antigen was bound to the plates in acidic or neutral conditions. Monoclonals 3H9, 1C3, 6E12, 4C6, and 3E8 reacted better with the antigen which was bound to the plates at neutral pH than with the antigen immobilized in acidic conditions, while monoclonals 2H5, 2E12, and 1G11 bound preferentially to the antigen immobilized under acidic conditions. We noted a striking correlation between the immunogen (i.e., whether the mice were injected with H5 or H5·tRNA) and the pattern of reactivity with acid or neutral pH immobilized H5. The antibodies which preferentially bind to H5 immobilized at neutral pH were elicited by injecting free H5 while the antibodies which preferentially bound to acid-treated H5 were elicited by H5·RNA complexes. The only antibody which did not show this correlation was antibody 3E8. Apparently the complexing of H5 with the nucleic acid brings about a conformational change which can be mimicked by treating the histone with acid. While these conformational changes cannot be detected with polyclonal antibodies, they are detectable by some of the monoclonal antibodies.

Competition Experiments Indicate Distinct Epitopes for Most Monoclonal Antibodies. The results presented so far suggest that the various monoclonal antibodies interact with distinct epitopes. To gain additional support for this notion and to determine the spatial proximity of the various epitopes, we performed competition experiments designed to test whether the binding of one monoclonal antibody will interfere with the binding of any other monoclonal. We reasoned that

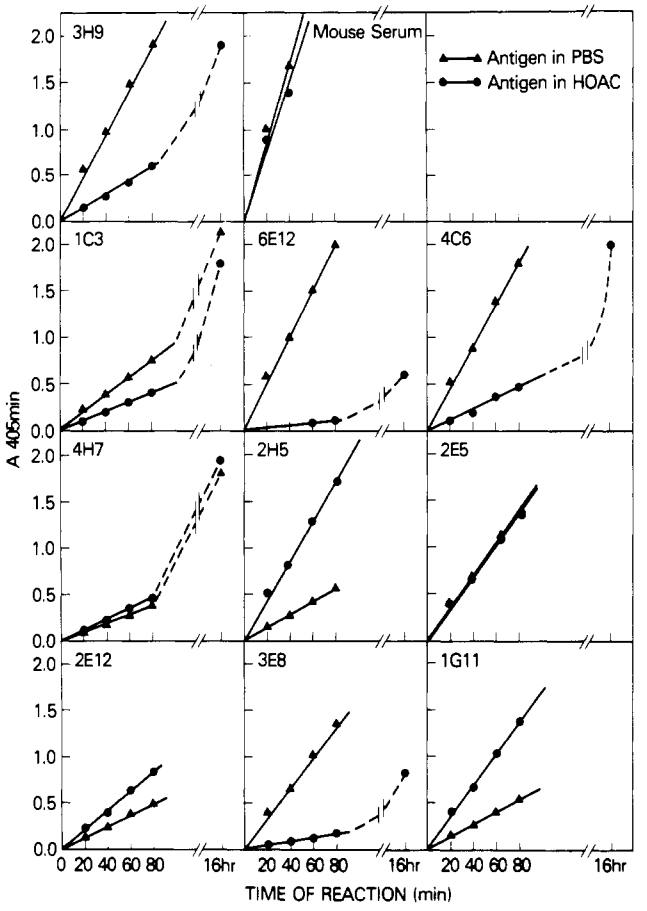

FIGURE 5: Effect of antigen conformation on antibody reactivity. Histone H5 was added to the microtiter plates, at 5 μg/mL, either in 0.1% acetic acid, pH 2.5, or in phosphate buffer, pH 7.0. The antigen was left on the plates for 18 h at 4 °C and the interaction with each monoclonal antibody determined by ELISA. In this experiment, the relative amount of antibody bound is indicated by the rate of substrate hydrolysis. Time of reaction refers to the time elapsed after the addition of substrate to the microtiter wells.

in the absence of steric hindrance the binding of antibodies to two distinct determinants should give an immunological reaction which is significantly higher than that obtained when only one type of antibody is bound to the molecule. To examine the feasibility of such an approach, we tested the ability of a polyclonal rabbit anti-H5 serum to inhibit the binding of several monoclonal antibodies. It was expected that the polyclonal antibody preparation, which presumably contains antibodies against all or most epitopes, should inhibit the binding of at least some of the monoclonal antibodies tested. The data presented in Figure 6A indicate that indeed the polyclonal antibodies inhibited the subsequent binding of mouse monoclonal antibodies. The ability of the rabbit serum to inhibit monoclonal antibody binding varied between the various monoclonals. Thus, at 1:20 serum dilution, the binding of monoclonal 3H9 was inhibited 100% while the binding of antibody 6E12 was inhibited only by 75%. The inhibitory capacity of the rabbit antiserum for a particular antibody probably depends on the amount of cross-reacting species in the rabbit serum and on the affinity constants of the rabbit and mouse antibodies.

The competition experiments involving pairs of monoclonal antibodies were performed at relatively low H5 concentrations so as to ensure that each of the monoclonals was present at antibody saturation. Each antibody was added to the antigen at various dilutions either as a separate component or as a mixture containing the same amounts of antibodies as each

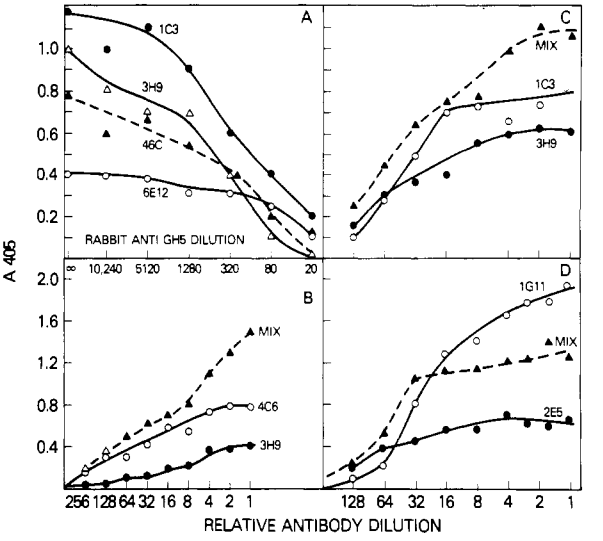

FIGURE 6: Competition experiments with pairs of monoclonal antibodies. (A) Polyclonal rabbit anti-H5 inhibits the subsequent binding of monoclonal antibodies. Monoclonal antibodies 1C3 (●), 3H9 (△), 4C6 (▲), and 6E12 (○) were mixed with various dilutions of polyclonal rabbit anti-H5 and added to microtiter plates containing H5. The relative amount of mouse monoclonal bound was determined by ELISA using alkaline phosphatase labeled goat anti-mouse Ig. Note that increasing the rabbit anti-H5 concentration decreased the amount of mouse Ig bound. (B–D) Competition experiments in which the monoclonal antibodies were added to the plates either as individual components or as a mixture. Note that in the absence of significant steric hindrance the mix always gives higher A_{405} values than for each monoclonal. This is most obvious at low dilutions, when all sites in H5 are saturated. In contrast, when mutual interference to binding is observed (D), the mix has higher values than those for each individual antibody at high antibody dilution, i.e., when the epitope is not fully saturated, and has an average A_{405} at low dilution.

separate antibody. Representative results are shown in Figure 6B–D. In Figure 6B, monoclonal antibody 3H9 reached saturation at 1:4 relative dilution. Doubling the antibody concentration to a 1:2 dilution did not give a higher A_{405} value. Antibody 4C6 reached saturation at 1:8 relative dilution. A mixture containing a 1:4 dilution of each of the above monoclonals gave a reaction significantly higher than each antibody separately, clearly indicating that the antibodies are bound to the antigen at distinct epitopes. A similar situation is presented in Figure 6C, where a mixture of monoclonals 1C3 and 3H9 gave a higher reaction than each separate antibody. In contrast, the binding of monoclonals 1G11 and 2E5 seems to be mutually exclusive (Figure 6D). A mixture of both antibodies displayed a binding curve which was the average of each individual antibody. Thus, monoclonals 1G11 and 2E5 constitute an antibody pair which apparently shares all or a part of the epitope. Competition experiments with other pairs of antibodies [see also Mendelson et al. (1984)] indicate that antibodies 1G11, 2E5, and 2H5 bind to overlapping epitopes while antibodies 1C3, 6E12, 4C6, 3H9, 2E12, 4H7, 3E8, and 2C8 bind to distinct, totally unrelated, epitopes.

The various characteristics of the monoclonal antibodies produced are summarized in Table II. Examination of this table clearly indicates that each antibody is directed against a distinct epitope. For example, 1G11 is distinguishable from 2E5 by its differential binding to peptide GH5 on nitrocellulose and monoclonals 1C3 and 6E12 by their cross-reactivity with H1°. The interaction of most antibodies with a unique epitope on H5 was verified by the competition experiments.

Exposure of Antigenic Determinants of H5 in Chromatin. Serological techniques are useful for elucidating the structure–function relation of chromosomal proteins (Bustin, 1979). Monoclonal antibodies which interact with distinct epitopes

Table II: Properties of Monoclonal Antibodies against H5

antibody:	1C3	6E12	4C6	3H9	2E5	1G11	2E12	4H7	2H5	3E8	2C8
immunogen:	H5	H5	H5	H5	H5·RNA	H5·RNA	H5·RNA	H5·RNA	H5·RNA	H5·RNA	H5·RNA
Ig type:	IgG_{2b}	IgG_{2b}	IgG_1	IgG_{2a}	IgG_1	IgM	IgM	IgG_3	IgM	IgM	IgG_1
reaction with H1°	+	–	–(+)[b]	+	–	–	–	–	–	–	+
reaction with H1	–	–	–	–	+	+	–	+	–	–	–
reaction with GH5 in ELISA	+	+	+	+	–	–	–	–	–	+	+
reaction with GH5 on nitrocellulose	+	–	+	+	+	–	+	+	+	NT[c]	NT
pH effect[a]	7 > 2	7 > 2	7 > 2	7 > 2	7 = 2	7 < 2	7 < 2	7 = 2	7 < 2	7 ≥ 2	NT

[a] pH effect refers to whether reaction at one pH was higher or lower than at the other; i.e., 7 > 2 indicates more antibody binding at pH 7 than at pH 2 while 7 < 2 indicates the opposite. [b] Monoclonal 4C6 reacted with H1° on nitrocellulose. The reaction was significantly weaker than with H5. [c] NT, not tested.

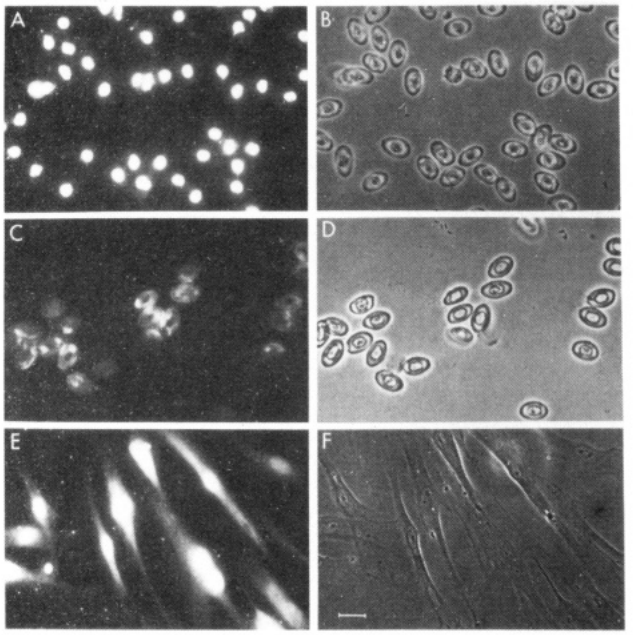

FIGURE 7: Indirect immunofluorescence of monoclonal antibodies with chicken erythrocytes and human KD fibroblasts. (A, B) Monoclonal 1C3 with chicken erythrocytes. (C, D) Control monoclonal with chicken erythrocytes. (E, F) Monoclonal 1C3 with KD human fibroblasts. Bars represent 20 m. (A, C, F) Fluorescence micrographs. (B, D, F) Corresponding phase-contrast micrographs.

allow investigations of the organization of specific, defined regions of the molecule in chromatin. For such studies, it is obligatory that the antigenic determinants in "native" chromatin are exposed and accessible to antibody binding. The ability of the various antibody molecules to recognize the antigenic determinants in native chromatin was tested by indirect immunofluorescence on chicken red blood cells and by binding studies to isolated chromatin. Each of the monoclonal antibodies displayed a strong nuclear fluorescence with chicken red blood cells fixed in methanol and acetone. Experiments with control monoclonal antibodies verified the specificity of the reaction (Figure 7). Monoclonals 1C3 and 3H9, which cross-react with H1°, gave positive immunofluorescence with human fibroblasts (KD cells) while the other monoclonals, which did not cross-react with H1°, failed to give positive immunofluorescence with the human fibroblasts. The immunofluorescence experiments, therefore, are in full agreement with the specificity studies which were done with the ELISA technique (Figure 3 and Table I). In addition, the experiments indicate that, in each cell, antigenic determinants against each type of antibody are exposed and available to interact with the antibody. These results do not necessarily mean that in chromatin each molecule of H5 is accessible to each type of monoclonal antibody. Since nucleosomes are heterogeneous (Goldblatt et al., 1978), it is possible that the various monoclonals interact with different types of nucleosomes.

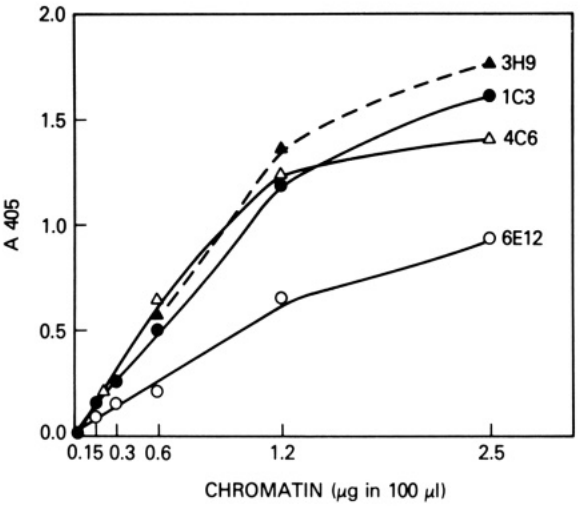

FIGURE 8: Monoclonal antibody binding to chromatin. Micrococcal nuclease digested chromatin, in 0.1 M NaCl, was added to the microtiter plates. The monoclonals were added as undiluted culture supernatants. The A_{405} values presented were obtained 3 h after substrate addition. The chromatin concentration is given in terms of DNA.

The monoclonal antibodies can bind to H5 not only in nuclei but also in isolated chromatin (Figure 8). Chromatin which was isolated from chicken red blood cells and mildly digested with micrococcal nuclease was used as the antigen in ELISA. Each monoclonal antibody tested bound to the isolated chromatin. Representative results are presented in Figure 8. We conclude that in purified chromatin a part of the H5 antigenic determinants is exposed and available to interact with the monoclonal antibodies produced.

Discussion

Immunochemical techniques have a unique advantage in the study of the structure–function relation of chromosomal proteins in that they enable the study of the organization of a particular component in the "native" nucleoprotein complex (Bustin, 1979). The usefulness of the serological approach is directly dependent on the availability of a large repertoire of specific antibodies directed against defined antigens. So far, most of the antibodies used in the study of chromatin have been elicited against intact purified proteins. Antibodies of narrower specificity have been generated by immunizing or adsorbing antisera with defined histone peptides obtained by cleavage of the intact molecule or by chemical synthesis (Muller et al., 1983).

With the aim of obtaining antibodies against distinct, defined regions of a chromosomal protein, we have elicited a series of monoclonal antibodies against histone H5, a protein which is involved in regulating the higher order structure of chromatin (Kornberg, 1977; McGhee & Felsenfeld, 1980). Previous serological studies indicated that the H5 molecule cross-reacts with histone H1°, a protein which seems to ac-

cumulate in differentiating or nonreplicating cells. We expected, therefore, that some of the monoclonal antibodies may serve as specific reagents for this very lysine-rich histone subfraction.

The relative high titer detected in all immunized animals regardless of whether they were immunized with H5 or H5·RNA complexes was expected since previous studies indicated that this histone is a good immunogen (Mura et al., 1980). While the type of Ig produced was not dependent on the immunogen, the specificity of the antibody seemed to be influenced by the conformation of the injected H5. Obviously, the small number of monoclonals examined does not allow statistical evaluations; however, we note that in the ELISA test, all antibodies obtained from mice immunized with uncomplexed H5 reacted strongly with the globular region of H5. In contrast, with the same assay, only two out of the seven monoclonal antibodies obtained from mice immunized with H5·RNA complexes reacted with GH5. In addition, two of the four antibodies obtained from H5-immunized mice reacted with H1°, while only one of the seven monoclonals obtained by H5·RNA immunization reacted with this protein. The reactivity toward calf thymus H1 also distinguishes between the monoclonals produced by H5 and H5·RNA. None of the H5-elicited monoclonals cross-reacted with this protein while three out of the five monoclonals elicited by H5·tRNA did. Finally, we note that exposure of H5 to low pH significantly diminishes the binding of the H5-elicited antibodies. In contrast, acid exposure had no effect or even enhanced the binding of the monoclonals elicited by H5·tRNA. Apparently the conformational effects occurring in H5 upon complexing with RNA are reflected in the specificity of the antibodies elicited.

During these studies, we were surprised to note that the binding of some of the antibodies to the antigens seemed to be dependent on the type of assay used to test the antibody. The two major assays used here were the ELISA and the immunoblotting assays; both involve interaction of antibodies with the antigen immobilized on a solid support. Yet antibody 6E12, which gave a strong positive reaction with GH5 in ELISA, failed to recognize the antigen when it was bound to the nitrocellulose sheet, while antibodies 2E5, 2E12, and 2H5 recognized the antigen (GH5) when bound on nitrocellulose but not when bound to the polystyrene plates used in the ELISA assays (see Table II). In each case, we verified that the GH5 molecule indeed was present on the nitrocellulose or in the microtiter plates by testing with another monoclonal. This observation suggests that the antigen does not bind to the solid support in a random manner. The binding of the protein to the solid support may result in a situation where a particular antigenic site is modified or sterically hindered in all the molecules. This situation has to be taken into account when monoclonal antibodies are used to detect and study the specificity of antigens.

All the monoclonal antibodies except monoclonal 1G11 reacted with the GH5 region of the molecule as determined either by ELISA or by immunoblotting. This region has already been identified as a major immunogenic epitope in the molecule (Mura et al., 1980). Examination of the information summarized in Table II indicates that the various antibodies reacted with different epitopes within the GH5 peptide. All the antibodies were distinguishable by either their cross-reactivity with H1° and H1, their binding to acid-treated H5, or their interaction with GH5 in the ELISA or immunoblotting assay. Competition experiments between various pairs of antibodies (Figure 6) further supported this observation. Thus, the data suggest that within the immunogenic GH5 region there is no epitope which is immunodominant in that a disproprotionately high number of cells produce antibody that interacts with it. However, competition experiments with antibodies 1G11 and 2E5 indicate that these antibodies react with identical or closely related epitopes. Indeed, precise mapping of the binding sites of these two antibodies indicates that both bind to residues 28–31 in the H5 molecule [see Mendelson et al. (1984)].

All the monoclonal antibodies produced and characterized recognize epitopes in chromatin-bound H5. The immunofluroescence results also indicate that the two monoclonals which cross-reacted with H1° recognized this protein in human KD fibroblasts. Thus, the monoclonal antibodies may be useful in various studies not only on histone H5 but also on the cellular role of H1°. The feasibility of such studies has already been demonstrated (Bustin, 1979).

Definition of the exact location of the epitope within the H5 molecule potentially would allow precise studies on the interaction of H5 with the core particle and follow putative rearrangement in chromatin structure associated with various functional changes in the cell. In the following paper (Mendelson et al., 1984), we use peptides obtained from H5 to map the binding sites of the various monoclonal antibodies on histone H5.

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Mapping the Binding of Monoclonal Antibodies to Histone H5†

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ABSTRACT: The binding sites of nine monoclonal antibodies along the polypeptide chain of histone H5 were mapped. Immunoblotting experiments with peptides generated from H5 by trypsin digestion, *N*-bromosuccinimide cleavage, and cyanogen bromide cleavage revealed that all of the monoclonal antibodies reacted with the globular region of H5 which is encompassed by amino acid residues 22-98. Within this globular segment, the epitopes could be subdivided into three regions. Monoclonals 1G11, 2E5, and 2H5 bind to residues 28-31. The close proximity of the epitopes was verified by a competitive enzyme-linked immunosorbent assay and by their binding pattern to a tryptic digest of H5. Monoclonals 4C6, 6E12, and 2E12 bind to a region encompassed by amino acids 28-53 while monoclonals 4H7, 1C3, and 3H9 bind to a region encompassed by residues 53-98. Precise localization of the epitopes in the primary sequence of H5 will allow detailed studies on the mode of binding of H5 to core particles in chromatin.

The lysine-rich histones stabilize the structure of the chromatosome and are essential for the formation of higher order chromatin structures (Thoma et al., 1979; Simpson, 1978; Renz et al., 1977; Butler & Thomas, 1981; Bradbury et al., 1978; McGhee & Felsenfeld, 1980). Mature chicken erythrocytes contain a lysine-rich histone variant, protein H5, which confers to the chromatin higher stability and transcriptional inactivity (Neelin et al., 1964; Kumar & Walker, 1980; Seligy et al., 1973; Sung & Freedlender, 1978; Thomas & Rees, 1983). A close analogue to histone H5, histone H1°, is found in cells showing little cell proliferation or in cells which are in the terminal states of differentiation [Pehrson & Cole, 1980; for further references on this topic, see Chabanas et al. (1983)]. It has been suggested that H5 exerts its specific biological effect by binding tightly to chromatin (Kumar & Walker, 1980; Thomas & Rees, 1983). The binding of H5 to chromatin is similar to that of the rest of the H1 class in that it depends on particular structural features of the molecule (Allan et al., 1980; Cary et al., 1981). Each member of the very lysine-rich class contains a folded central domain consisting of 80 amino acids which locates the molecule in chromatin, most probably at the exit-entry points of the 160 base pair chromatosomal DNA.

The exact manner in which the molecule binds to DNA and exerts its structural effects is not fully known. Serological techniques can be used for various structural studies on chromosomal proteins (Bustin, 1979). More meaningful interpretation of immunochemical data, however, requires exact definition of the epitopes which are recognized by various antibodies. With the aim of elucidating the mode of binding of H5 to chromatin, we have elicited and characterized a series of monoclonal antibodies specific for distinguishable epitopes [see the preceding paper in this issue (Mendelson & Bustin, 1984)]. In the present paper, we further characterize the antibodies, and we map the location of the epitopes along the polypeptide chain of histone H5.

Materials and Methods

Antigens and Antibodies. The preparation of the antigens and the production and characterization of the monoclonal antibodies have been described in the preceding paper (Mendelson & Bustin, 1984).

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