Differing Accessibility in Chromatin of the Antigenic Sites of Regions 1–58 and 63–125 of Histone H2B

DIANA DI PADUA MATHIEU, CASILDA V. MURA, LING-LING Y. FRADO, CHRISTOPHER L. F. WOODCOCK, and B. DAVID STOLLAR
Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111; and the Department of Zoology, Morrill Science Center, University of Massachusetts, Amherst, Massachusetts 01003

ABSTRACT Experiments with antibodies induced by separated fragments 1–58 and 63–125 of H2B histone indicated that the 1–58 portion of the molecule is much more accessible in chromatin than is the 63–125 region. In immunoabsorption and immunoelectron microscopic assays with bovine and chicken chromatins, anti-1–58 antibodies reacted with sheared or unsheared chromatin both at low ionic strength (1 mM Tris-HCl) and in 0.14 M NaCl. Anti-63–125 antibodies were bound only weakly by chromatin at low ionic strength and not at all in 0.14 M NaCl. Antibodies to whole H2B showed intermediate reactivity with chromatin in both assays. In tests of immunofluorescence with unfixed calf liver nuclei in suspension, anti-1–58 caused nucleolar as well as nucleoplasmic fluorescence, whereas anti-63–125 did not lead to detectable fluorescence; anti-H2B showed intermediate staining intensity. In control experiments, anti-H1 antibody was bound by chromatin at low ionic strength but not in 0.14 M NaCl; anti-H3 antibody was bound poorly under either condition.

It has been established that two molecules of each of the histones H2A, H2B, H3, and H4 constitute a core for nucleosome subunits of chromatin and that DNA is wrapped around the outside of the core (19). The precise pathway of the DNA, the nucleosome topography, and the DNA-histone interactions are still under study. In one approach to these questions, exposure of chromatin or nucleosomes to trypsin led to the selective cleavage of the amino terminal 20-30 amino acids of each of the core histones (30), suggesting that these regions are exposed under the conditions of digestion (usually low ionic strength, such as 5 mM Tris-HCl, pH 8). The rate of cleavage was most rapid for H3. Progressively slower cleavage occurred with H2A, H4, and H2B (31). The interpretation that this susceptibility to trypsin reflects simply selective exposure of the amino terminal regions at the nucleosomal surface or their extension from the core, however, has been questioned (18).

Antibodies provide another kind of probe for exploring the organization of histones in chromatin and nucleosomes (7, 25). Studies in which chromatin was used as an immunoabsorbent showed that many of the antigenic sites that are recognized in separated core histones are masked in chromatin; this is especially true for H2A, H3, and H4 determinants, whereas those of H1 and H2B are relatively accessible (6, 10, 11). Different antigenic determinants, therefore, are exposed to varying extents in chromatin or altered in conformation to varying degrees.

The accessibility of some antigenic sites of H2B in nucleosomes, at least in solutions of low ionic strength, was also demonstrable by measurement of a specific increase in the sedimentation rate of nucleosomes after their incubation with purified anti-H2B antibodies (1, 24). Further, anti-H2B antibodies that bound to nucleosomes or chromatin have been detected by immunoelectron microscopy (8), and injection of anti-H2B antibodies into amphibian oocytes resulted in marked changes in the structure of transcription complexes (23).

The value of immunological experiments can be enhanced by the use of antibodies directed against a selected portion of the histone molecule. We recently examined the distribution of antibodies in an anti-H2B antiserum and found that 90% of the antibody was directed against the carboxyl terminal half of the molecule (9). The question was raised whether that part of the molecule was being measured in the above experiments, or whether the results depended largely on the smaller antibody population directed against the amino terminal half of the molecule. Absolom and Van Regenmortel (1), for example, found that a fraction of anti-H2B antibodies that reacted with the peptide fragment 30–56 of H2B were bound by nucleosomes as measured by immunosedimentation. We have pre-

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pared separate antisera against the N-terminal and C-terminal fragments, 1–58 and 63–125, produced by cyanogen bromide cleavage of H2B (9), and have now used the antibodies to measure the accessibility of these two parts of the molecule in native chromatin. Reactions were measured by immunoabsorption, immunofluorescence, and immunoelectron microscopy.

MATERIALS AND METHODS

Antigens and Antisera

H1, H2B, and H3 histones were prepared from calf thymus by the procedure of Johns (16) and further purified by gel filtration on Bio-Gel P-60 columns (Bio-Rad Laboratories, Richmond, Calif.). The columns were equilibrated and developed with 0.02 N HCl, 50 mM NaCl, and 0.02% sodium azide. Fragments 1–58 and 63–125 of H2B were prepared by cleavage of H2B with cyanogen bromide in formic acid (17); the fragments were purified and characterized previously (9). The antisera, induced by immunization of rabbits with histone-RNA complexes, have been described (9, 27). IgG fractions of antisera were prepared by DEAE-cellulose chromatography (26).

For immunoelectron microscopy, antibodies were specifically purified by affinity chromatography. The IgG fractions of antisera induced by H2B, and by fragment 1–58, were purified with columns of H2B-Sepharose; anti-63–125 was purified with a 63–125-Sepharose column (9). The columns were washed with phosphate-buffered saline (PBS), borate-buffered saline (0.125 M sodium borate pH 8.5, 1 M NaCl, 0.1% Tween-20), and again with PBS. Antibody was eluted with 4 M guanidinium hydrochloride, pH 8, and dialyzed against PBS. Specificity of purified antibodies was tested by complement fixation and solid-phase radioimmunoassay. Immunospecifically purified anti-H2B and anti-63–125 and the IgG of anti-1–58 were used for immunofluorescence.

Chromatin

Sheared calf thymus chromatin was prepared as described by Zubay and Doty (33) either from fresh thymus or from tissue stored frozen at −70°C. The thymus tissue was homogenized in 5 vol of 0.075 M NaCl–0.025 M EDTA (pH 8) in a Waring blender (Dynamic Corp. of America, New Hartford, Conn.), strained through cheesecloth, and centrifuged. The pellet was resuspended and washed several times with the same solution until the supernatant fluid reached a minimal content of ultraviolet-absorbing material. This chromatin was either used immediately or stored in 50% glycerol at −20°C.

Unsheared chromatin was prepared by brief exposure of isolated nuclei to micrococcal nuclease. Nuclei were prepared from calf thymus by the procedure of Holman and Deicher (14) and incubated with micrococcal nuclease (0.3 U [10 ng]/A_{260} U of chromatin, with 1 mM $CaCl_2$) for 1 min at 37°C. EDTA was added to a final concentration of 10 mM and the reaction mixture was chilled on ice. It was centrifuged at 2,000 rpm for 10 min and the supernatant fluid retained. About 60% of the DNA was present in the supernate. This chromatin was kept at 4°C and used immediately or the next day.

Serological Assays

Quantitative microcomplement fixation reactions were performed as described by Stollar (25), and solid-phase radioimmunoassays as described by Blankstein et al. (3). Immunoabsorption assays (11) were performed with the IgG fractions of anti-H2B, anti-1–58, and anti-63–125 sera, and with whole anti-H1 and anti-H3 antisera. The antibodies were incubated with buffer alone or with sheared or unsheared chromatin containing 25 or 125 μg of DNA under conditions of low ionic strength (1 mM Tris-HCl, pH 8) or physiologic ionic strength (0.14 M NaCl, 0.01 M Tris-HCl, pH 8). Each serum or IgG sample was pretitrated and the dilution used for absorption was one that was expected to result in 70 to 80% complement fixation when the mixture was diluted 120-fold further in the final serological assay. These dilutions were 1:10 to 1:20 for IgG fractions and 1:100 to 1:200 for whole sera. The absorption mixtures were incubated for 1 h at 37°C and for 4 h at 4°C. After this incubation, the mixtures in hypotonic buffer were brought to 0.14 M NaCl and 0.02 M sodium phosphate (pH 6.8) by the addition of 1 vol of a stock solution twice that concentration. 1 vol of the same stock solution was also added to the mixtures in buffered saline. After an additional incubation of 15 min at 4°C, all samples were centrifuged at 5,000 *g* for 30 min; at least 95% of the chromatin was pelletted. The supernates were then diluted into complement fixation assays to measure residual antibody activity.

Immunofluorescence

Bovine liver nuclei were prepared from fresh liver by a modification of the method of Blobel and Potter (4) with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) present throughout. From the crude nuclear pellet, the lower layer containing nuclei and erythrocytes was removed and resuspended in 50 mM Tris HCl, 25 mM KCl, 5 mM $MgCl_2$ (TKM) buffer (pH 7.5) containing 0.2% Nonidet P40 detergent. Stirring at room temperature for 10 min effectively lysed erythrocytes and whole hepatocytes, and the nuclei were then pelleted at 350 *g* for 10 min. After further washing, the pellet, consisting mainly of well-preserved nuclei, was resuspended in 0.15 M NaCl, 0.1 mM PMSF. Chicken erythrocyte nuclei were prepared from fresh blood as described previously (22).

For immunofluorescence preparations, ~10^6 nuclei in 10 μl of 0.15 M NaCl, 0.1 mM PMSF were incubated for 30 min on ice with 2–8 μg of affinity-purified anti-H2B or anti-63–125, or 20 μg of anti-1–58 IgG antibody in 10–40 μl of PBS. The suspension was then centrifuged for 4 min (Eppendorf model 5412, Brinkmann Instruments, Inc., Westbury, N. Y.), and the nuclear pellet was washed once with saline-PMSF and then resuspended in 20 μl of a 10- to 30-fold dilution of fluorescein-conjugated goat anti-rabbit IgG (N.L. Cappel Laboratories, Inc., Cochranville, Pa.). After a further 30-min incubation, the nuclei were washed again, resuspended in 20 μl of saline-PMSF, and placed on a glass microscope slide.

Alternatively, nuclei were attached to slides and fixed before reacting with antibody. In this case, nuclear suspensions were smeared over the surface of slides that had been pretreated with an aqueous solution of 1% (wt/vol) ficoll. Slides were then immersed, at room temperature, in 100% ethanol for 5 min, followed by immersion in acetone for 2 min, and then air dried. After rehydration in PBS for 5 min, a 20-μl drop containing 4 μg of antibody in PBS was placed on each slide, a coverslip was added and the sample incubated for 1 h at room temperature in a humid chamber. The coverslip was then removed, and the slide washed twice with PBS and incubated as before with fluorescein-conjugated goat anti-rabbit antibody. After a second series of washes, the slides were examined with a Zeiss photomicroscope equipped with epifluorescence optics. Photomicrographs were taken on Tri-X pan film and processed with Diafine developer.

Electron Microscopy

In general, the procedures of Miller and Bakken (21) for spreading chromatin and of McKnight et al. (20) for immunoferritin labeling were followed. Washed chicken erythrocytes in 0.15 M NaCl (pH 8.0) were pelleted lightly and resuspended in saline at a 1:1 (vol/vol) ratio. Lysis was achieved by adding 5 μl of cell suspension to 1.5 ml H_2O adjusted to pH 9.4 with borate buffer. After standing for 10 min at room temperature, 20-30 μl of the lysate was incubated for 10 min with 0.2-2.0 μg of affinity-purified antibody at room temperature, after which 5–10 μl (O.D. 410 = 1.38) of ferritin conjugated to goat anti-rabbit IgG (Miles Laboratories, Elkhart, Ind.) was added for a further 5–10 min. The ferritin conjugate had previously been passed through a Sepharose 2B column equilibrated with 10 mM NaCl, 1 mM triethanolamine HCl, 0.2 mM EDTA (pH 8.0) to purify the monomeric form (32). To prepare the immunoreacted chromatin for electron microscopy, aliquots were centrifuged through a cushion of 0.1 M sucrose, 10% formalin onto a freshly glow-discharged carbon film. The supernatant fluid was repeatedly withdrawn from the micro chamber and replaced with pH 4 H_2O to remove unbound ferritin, after which the grid was removed, stained with 2% aqueous uranyl acetate, and examined with a Siemens 102 electron microscope operated at 60 KV.

RESULTS

Immunoabsorption

Anti-H1 and anti-H3 antisera was used as standards for measuring maximal and minimal absorption of antibody by chromatin. The exposure of determinants for these sera, however, varied with experimental conditions and the source of chromatin. As previously reported (10), 90% or more of the serological activity of anti-H1 was absorbed by small amounts of either sheared or unsheared chromatin when the absorption was performed at low ionic strength (Table I). In isotonic buffer, however, neither the sheared nor unsheared chromatin absorbed anti-H1 antibodies (Table II). With anti-H3 serum, in agreement with previous results (10, 11), little antibody was absorbed by calf thymus chromatin, whether it was sheared or not (Table I). We have since found, as have Goldblatt and Bustin (12), that mouse liver chromatin is more active than calf thymus chromatin in absorbing anti-H3.

Experiments were then carried out with antibodies to H2B and to its fragments 1–58 and 63–125. The specificities of these

TABLE I

Absorption of Antihistone Antibodies by Calf Thymus Chromatin at Low Ionic Strength

Serum	Chromatin	DNA content	Decrease in peak percentage of C fixation*	
		μg	%	
Anti-H1 (88)‡	Sheared	25	90	
		125	95	
	Unsheared	25	93	
		125	100	
Anti-H3 (92)‡	Sheared	25	2	
		125	17	
	Unsheared	25	4	
		125	4	
Anti-H2B			H2B (86)‡	
	Sheared	25	92	
		125	81	
	Unsheared	25	87	
		125	90	
Anti-(1-58)			H2B (44)‡	(1-58) (92)‡
	Sheared	25	100	92
		125	100	100
	Unsheared	25	100	82
		125	100	100
Anti-(63-125)			H2B (46)‡	(63-125) (98)‡
	Sheared	25	0	0
		125	68	0
	Unsheared	25	0	0
		125	66	0

* Anti-H1 and anti-H3 were tested with homologous histones. Anti-H2B, anti-1-58, and anti-63-125 were tested with H2B or homologous fragments as indicated.
‡ Peak percentage of C fixation of the control, unabsorbed serum sample is shown in parentheses. Although these values vary from 44 to 98%, they represent an amount of antibody activity that is within a twofold dilution of the C fixation endpoint.

TABLE II

Absorption of Antihistone Antibodies by Calf Thymus Chromatin in 0.14 M NaCl

Serum	Chromatin	DNA content	Decrease in peak percentage of C fixation	
		μg	%	
Anti-H1 (50)*	Sheared	25	12	
		125	10	
	Unsheared	25	30	
		125	0	
Anti-H2B			H2B (52)*	
	Sheared	25	46	
		125	42	
	Unsheared	25	0	
		125	38	
Anti-(1-58)			H2B (86)*	(1-58) (96)*
	Sheared	25	68	85
		125	86	100
	Unsheared	25	71	40
		125	93	92
Anti-(63-125)			H2B (98)*	(63-125) (78)*
	Sheared	25	0	0
		125	0	0
	Unsheared	25	0	0
		125	0	0

* Peak percentage of C fixation of the control, unabsorbed serum sample is shown in parentheses.

antisera were studied previously (9). Anti-H2B IgG reacted with H2B more strongly than with either fragment. About 90% of the anti-H2B antibody was isolated by a 63–125-Sepharose affinity column and 10% by a 1–58-Sepharose column. For the studies described here, antisera were induced separately by 1–58 and 63–125 RNA complexes (9). In complement fixation experiments, early bleeds of anti-1–58 reacted with intact H2B and with 1–58, but not with 63–125; later bleeds reacted with 1–58 but not with intact H2B or with 63–125. An early serum, reactive with H2B, was chosen for the present experiments. The anti-63–125 serum reacted more strongly with H2B than with 63–125 itself and did not react with 1–58. Anti-1–58 recognized large differences between calf H2B and H2B of *Drosophila*, sea urchin, or trout, whereas anti-63–125 reacted nearly identically with all of them. These results were in accord with data on amino acid sequences of these proteins (15).

At low ionic strength (1 mM Tris-HCl), 25 μg of sheared or unsheared chromatin absorbed nearly all the serological reactivity of anti-H2B, as tested with H2B antigen (Table I). In 0.14 M NaCl, absorption was much less effective, especially

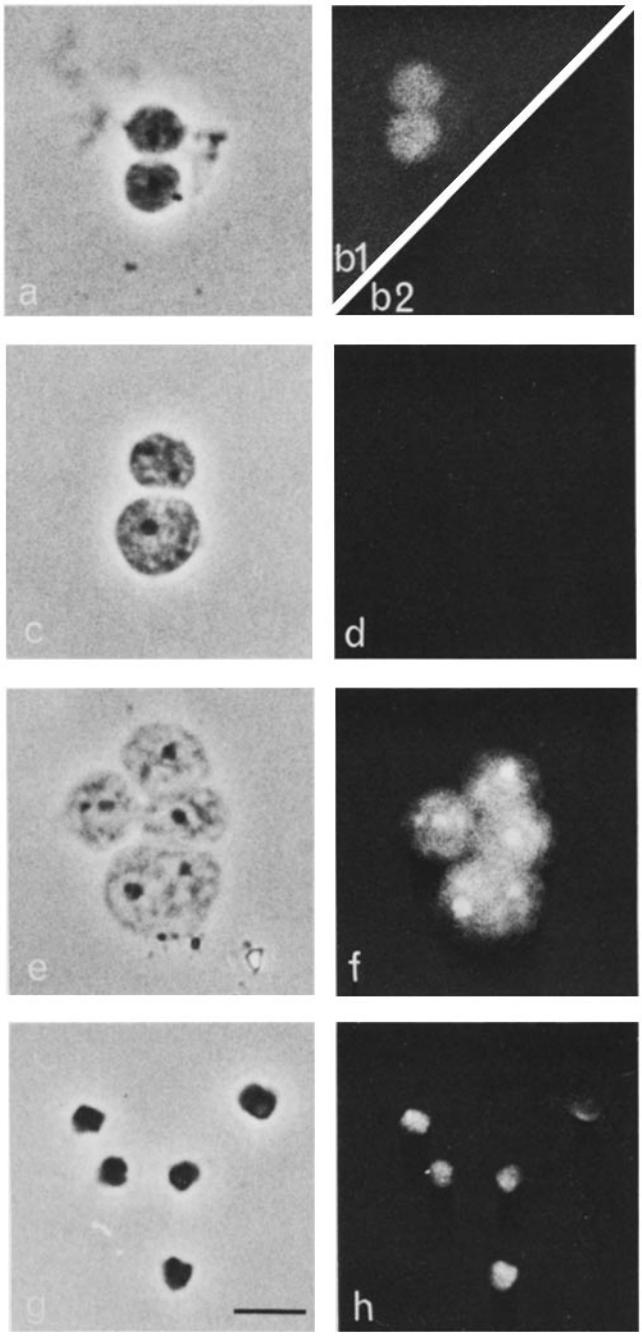


FIGURE 1 Immunofluorescence with affinity-purified antibodies to H2B and its fragments 1-58 and 63-125. Phase microscopy pictures of bovine liver nuclei (*a*, *c*, *e*) and chick erythrocytes nuclei (*g*). Immunofluorescence with antibodies to: whole H2B (*b1* printed with short exposure, *b2* with standard exposure); anti-63-125 (*d*); and anti-1-58 (*f*, *h*). Bar, 10 μm.

with unsheared chromatin (Tables I and II). When tested with 63–125 as antigen, unabsorbed serum reacted to give a lower extent of complement fixation than it did with whole H2B, but most of the reactivity persisted after absorption with 25 μg of chromatin at low ionic strength; even 125 μg of chromatin did not absorb all of it.

A marked difference in antibody absorption was observed when antibodies to the two halves of the H2B molecule were compared. Antibodies to the N-terminal fragment 1–58 were readily absorbed by 25 or 125 μg of sheared or unsheared chromatin at low ionic strength. This was evident whether the residual antibodies were tested with H2B or with the fragment itself (Table I). In 0.14 NaCl, determinants on the amino terminal half of the molecule were still accessible in chromatin; however, more chromatin was required for nearly complete absorption (Table II), especially with the unsheared preparation. Thus, some of the fragment I–58 determinants were masked under these conditions.

In contrast to the above findings, there was no absorption of anti-63–125 antibodies by sheared or unsheared chromatin in 0.14 M NaCl (Table II) and very little absorption in low ionic strength (Table I). In the latter case, no loss of the strong homologous reaction with 63–125 was observed after incubation of serum with 125 μg of chromatin; only partial decrease of a weaker reaction of more dilute serum with whole H2B indicated any immunoabsorption (Table I).

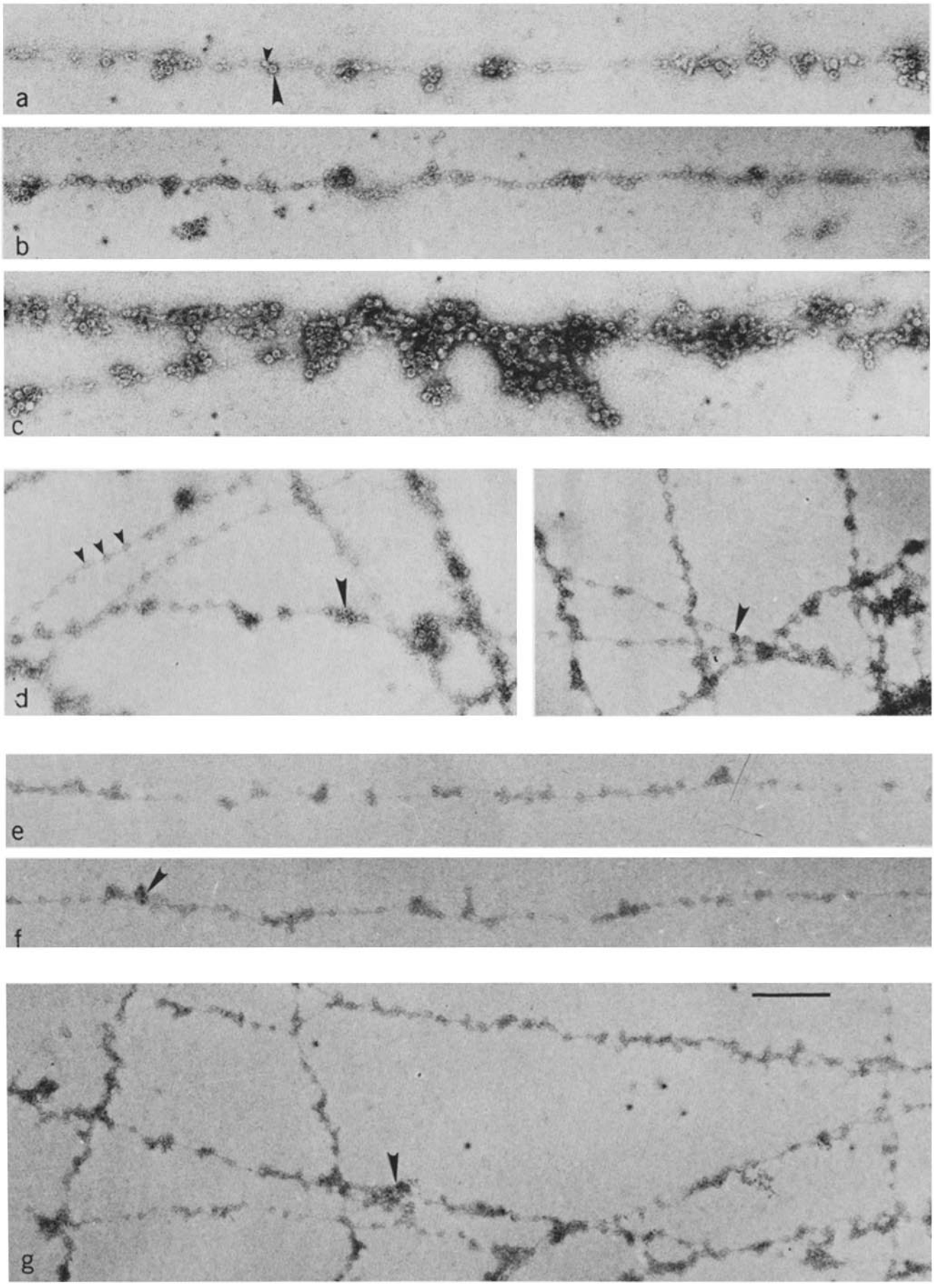
Immunofluorescence

Antibodies raised against whole calf thymus H2B bound to bovine liver nuclei, as determined by indirect immunofluorescence with suspended unfixed nuclei (Fig. 1 *b*). A much stronger reaction was seen when approximately the same amount of anti-1–58 antibody was used (Fig. 1*f*). Under standard conditions of photographic printing, this staining was clearly visible (Fig. 1*f*) whereas the anti-whole H2B staining was not (Fig. 1*b2*). A shorter printing exposure allowed recording of the weaker anti-H2B reaction (Fig. 1*b1*). With anti-1–58 antibody, used at appropriate dilution, nucleolar fluorescence stood out as brighter than that of the nucleoplasm (Fig. 1*f*). A similar nucleoplasmic and nucleolar fluorescence was caused by anti-H1 antiserum with unfixed nuclei (data not shown). In contrast to these results, anti-63–125, used at the same concentration, gave no detectable fluorescence (Fig. 1*d*); this result was negative with either the standard or shortened photographic printing exposure. Anti-1–58 also stained chick erythrocyte nuclei (Fig. 1*h*) more brightly than did anti-H2B (not shown); anti-63–125 was again negative in this case. Similar results were obtained with unfixed nuclei in suspension and with ethanol-fixed nuclei attached to slides.

Immunoelectron Microscopy

The same pattern of reaction as described above was observed at the ultrastructural level. Incubation of dispersed

FIGURE 2 Immunoelectron microscopy of chromatin with affinity-purified antibodies to H2B and to its fragments 1-58 and 63-125. Chromatin dispersed upon gentle lysis of chick erythrocyte nuclei was reacted with anti-1-58 (*a*, *b*, *c*), anti-whole H2B (*d*), anti-63–125 (*e*, *f*), or no antibody (*g*) and then with ferritin-conjugated anti-rabbit IgG. The reacted chromatin was then centrifuged through a cushion of 0.1 M sucrose, 10% formalin, onto a carbon film. Unbound ferritin was removed and the grid was stained with aqueous uranyl acetate. The small arrowheads identify nucleosomes; the large arrowheads identify ferritin. Bar, 100 nm.


a
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erythrocyte chromatin with anti-1–58 followed by ferritin-conjugated second antibody frequently resulted in extensive decoration with ferritin (Fig. 2 *a*, *c*), often obscuring the underlying nucleosomes (Fig. 2 *c*). Antibody to whole H2B produced an intermediate level of binding (Fig. 2 *d*), whereas anti-63–125 gave only a background level of binding (Fig. 2 *e*, *f*), similar to that obtained with the second antibody-ferritin conjugate alone (Fig. 2 *g*).

DISCUSSION

These experiments demonstrate that the amino-terminal portion of H2B is accessible in chromatin, in a serologically reactive conformation similar to that in isolated H2B. The carboxyl-terminal half of the protein, however, is either altered in conformation within chromatin or, more likely, masked by histone histone and histone DNA interactions. The usefulness of the antibodies as probes for these regions of the molecule depend on the previously described specificities of the antisera (9).

These conclusions were suggested first when anti-H2B IgG was absorbed with chromatin and residual activity was tested with either H2B or fragment 63–125. The absorption reduced the reactivity of this serum with whole H2B much more readily than its reactivity with 63–125. At the serum dilution used, the aggregation required for complement fixation with H2B probably involved both the minor anti-1–58 population and a similar amount of the anti-63–125 antibodies; the latter would be only a small portion of the total anti-63–125 population, so that the complement fixation reaction would be especially sensitive to a decrease in the limiting anti-1–58 antibodies. When the control reaction depended only on anti-63–125, absorption led to much less reduction of complement fixation.

The conclusions were confirmed in a more definitive analysis undertaken with antibodies that were specific for each fragment and still reactive with intact H2B. It was previously shown that the anti-1–58 serum reactions were very sensitive to evolutionary sequence changes that occur in the 1–34 region (9), so that at least some of its antigenic determinants are in, or are dependent on, that region.

The present absorption experiments were carried out at low ionic strength, as were those of Goldblatt and Bustin (11), and in 0.14 M NaCl as well. In the former case, the nucleosomes would be expected to be partially unfolded (13) and the higher order structure relaxed. It was at low ionic strength that some of the 63–125 determinants of chromatin were accessible to antibody. In 0.14 M NaCl, the chromatin was insoluble and was used as a very finely dispersed suspension; this would hinder uniform access of antibody to some portions of the chromatin and would measure primarily structures exposed at the surface of the particles. In this condensed state the 63–125 determinants were not available but the 1–58 region still reacted with antibody. More chromatin was required for removal of the anti-1–58 antibody in 0.14 M NaCl than in 1 mM Tris, probably reflecting a partial masking even of this region in the condensed state. A particularly interesting finding was the marked difference in absorption of anti-H1, the standard for effective reaction with chromatin at low ionic strength. As expected, it was readily removed in the experiments in 1 mM Tris-HCl but was not absorbed in 0.14 M NaCl. This finding agrees with the results reported by Takahashi and Toshira (28) and is consistent with the proposal of Thoma et al. (29) that, in the higher order structure of condensed chromatin, H1 is in the center of superhelical structures, where it would not be accessible to antibody. On the other hand, anti-H1 antibody does cause immunofluorescent staining of nuclei under most conditions of fixation, indicating that some of the H1 is accessible *in situ*, and Allan et al. (2) have found that H1 within nuclei is susceptible to digestion by trypsin. The results with H1 raise the question of whether its location and conformation are comparable in nuclei and in chromatin particles in 0.14 M NaCl.

The immunofluorescence and electron microscopy experiments also confirmed the greater accessibility of antigenic determinants of the 1–58 portion of H2B. Immunofluorescence was performed under isotonic conditions with suspended nuclei. This allowed the study of bovine liver nuclei without variations due to tissue sectioning or fixation. The nuclei were treated with nonionic detergent during preparation and were readily penetrated by antibodies. Similar results were obtained with nuclei fixed to slides with ethanol and acetone. Staining of isolated nuclei of chicken erythrocytes was much more uniform than staining of nuclei in fixed whole cells, probably due to more uniform penetration of isolated nuclei by antibody. With anti-1–58 and anti-H1 IgG, the nucleolar staining was surprisingly prominent. This may reflect a partial decondensation of the chromatin that extends into the nucleoli, because the condensation of DNA within nucleoli is less than in nucleoplasm (5). Alternatively, because the animals were immunized with histone-RNA, this reaction could be due to a histone-nucleic acid complex; the sera do not show specific reactivity with free RNA in complement fixation or radioimmunoassays.

Immunoelectron microscopy measured binding at low ionic strength by chromatin prepared simply by gentle lysis of nuclei, with minimal shearing. Immunospecifically purified antibodies were used in all cases. Anti-1–58 reacted strongly whereas anti-63–125 did not bind detectably to this chromatin.

These experiments demonstrate the increased resolving power of antibody reagents directed against selected portions of a protein antigen in comparison with antibodies to the whole protein. Even more narrowly focused antibody probes may be prepared either by the further absorption and affinity purification with smaller fragments of the protein or by the preparation of monoclonal antibodies of hybridoma origin.

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Reprint requests should be addressed to Dr. Stollar at Tufts University School of Medicine.

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