Mapping of Histone H5 Sites on Nucleosomes Using Immunoelectron Microscopy*

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The location of histone H5 on nucleosomes has been determined by binding anti-H5 antibodies to dinucleosomes, and recording the position of the bound IgG molecules using electron microscopy. Two types of antibody were employed, a total IgG fraction prepared from rabbits immunized with H5/RNA, which reacted to all three domains (NH2-terminal, central globular, and COOH-terminal) of H5, and an immunospecific fraction which bound only to the central globular peptide. After reacting with dinucleosomes, both types of antibody were localized primarily in the linker DNA entry/exit region, but the whole antibody showed a much greater affinity for the linker DNA itself than did the antiglobular peptide antibody. These results provide direct support for the concept that H5, and by inference H1, is located at the linker DNA entry/exit site of the nucleosome, and further suggest that it is the central globular portion of the molecule that is most closely associated with this site. An interaction of one or both termini of H5 with the linker DNA is also indicated.

In most eukaryotes, the genetic material is organized into repeating structures, the nucleosomes, in which the DNA is distributed between core and linker regions. The nucleosomal core comprises ~145 bp of DNA wrapped in 1.75 turns around an octamer of histones which is made up of two each of the core histones H2A, H2B, H3, and H4. Linker DNA of variable length connects the core particles, and it is with the linker that the very lysine-rich histones seem to be associated (for reviews of chromatin structure, see Refs. 1 and 2). The class of very lysine-rich histones includes H1, which often occurs as several variants, H1*, and the nucleated erythrocyte-specific H5. These histones are characterized by a conserved central globular domain flanked by more variable and extended NH2- and COOH-terminal regions (reviewed in Ref. 3). H1 (H5) is required for the orderly condensation of the ~10-nm diameter nucleosomal fiber into the ~30-nm fiber which is typical of nuclear chromatin (4, 5). In order for transcription and replication to occur, it is probably necessary for the 30-nm fiber to enter a programmed cycle of unfolding and refolding, processes in which H1 must be intimately involved, and thus an important factor in understanding these processes is the location of H1 (H5) in chromatin. As discussed below, several indirect lines of evidence strongly suggest that these histones bind to the entry/exit site of the linker DNA, thus sealing two complete turns around the nucleosome (3, 5–8). Using direct visualization of anti-H5 antibodies bound to nucleosomes, we have been able to confirm this location. It has also been possible to record differences in the location of bound IgG between preparations containing antibodies against all three domains of H5 (globular, NH2-terminal, and COOH-terminal peptides) and an immunospecifically purified fraction directed against the central globular region only.

Although H1 and H5 appear to occupy equivalent sites in chromatin, they show marked differences in binding affinity (34) such that H1 readily redistributes itself even in low ionic strength solutions, while H5 is stable at least up to 80 mM NaCl (35, 36). The stronger binding of H5 allows antibody probes to be used with more confidence than would be possible with H1.

MATERIALS AND METHODS

Histone and Antibody Preparation—Histone H5 was prepared from erythrocytes of adult white leghorn chickens (9) and its purity was assayed by SDS-polyacrylamide gel electrophoresis (10). For preparation of the globular segment of H5, the purified protein was dissolved in 0.2 M K2SO4, 50 mM Tris/HCl buffer, pH 8.0, at a concentration of 20 mg/ml. Trypsin was added at an enzyme to substrate ratio of 1:1000 and the digestion carried out at 20 °C for 120 min (10). The reaction was quenched with 0.02% of 1-chloro-3-tosylamido-7-aminohexane and the mixture was loaded onto a column of Sephadex G-50F. The elution was carried out with 20 mM HCl, 0.5 M NaCl. The chromatographic fractions were desalted by dialysis and recovered by acetone precipitation. Amino acid analysis showed that the peptide comprised amino acids 22–100.

The acetic acid fragments of H5 were prepared by hydrolysis with 0.2 N acetic acid at 105 °C in an evacuated sealed tube for 6 h. The hydrolysate was lyophilized and dissolved in 0.01 N HCl (22).

Antibodies against H5 were prepared from sera of rabbits immunized with H5 complexed with RNA in a 3:1 ratio (11). Each animal received 50 μg of complexed H5 dissolved in isotonic 0.9% NaCl solution. Antibodies were detected and characterized by solid phase radioimmunoassay as modified for the use of polyvinyl microtiter plates (12). IgG was purified from each serum by DEAE-cellulose chromatography.

Purification of Antibodies—Anti-H5 antibodies were immunospecifically purified from IgG by affinity chromatography with H5 conjugated to Sepharose 4B.

Electrophoretic Transfer of Protein to Nitrocellulose Paper—Histones and peptides were analyzed by gel electrophoresis as described above. The electrophoretic transfer of proteins from the polyacrylamide gel to nitrocellulose paper (Schleicher & Schuell) was done at

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The abbreviations used are: bp, base pair; SDS, sodium dodecyl sulfate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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10 V for 2 h using an electrophoretic apparatus (E-C Apparatus Corp., St. Petersburg, FL) as described by Towbin et al. (14).

Electrophoretic blots were soaked in 3% bovine serum albumin in Tris/saline (0.14 m NaCl, 1 mM Tris-HCl, pH 7.0) at 4 °C overnight. They were washed with Tris/saline and incubated with 3 ml of the appropriate antibody dilution (200 μg of purified rabbit anti-H5/RNA and 150 μg of purified rabbit antiglobular H5). After 2 h at room temperature, the blots were washed three times for 10 min with the following solutions in succession: Tween/BSA (0.1 m boric acid, 0.025 m sodium borate, 1 mM NaCl, 0.1% Tween 20, pH 8.5), Tween/saline (0.5% Tween 20 in 0.15 m NaCl), and Tris/saline. The washed blots were incubated with 5 ml of 125I-protein A in 3% bovine serum albumin containing 2 × 10^6 cpm/ml for 1 h at room temperature. The blots were washed as indicated above, thoroughly dried, and exposed to Kodak X-Omat R film.

Preparation of Dinucleosomes—Dinucleosomes from chicken erythrocyte nuclei were obtained as previously described (15) with the following modifications. Nuclei were digested with micrococcal nuclease for 7 min in 1 mM CaCl_2, 1 mM Tris, pH 8.0, using 0.5 unit of enzyme ( Worthington) per 1.0 A260 of chromatin at 37 °C. The reaction was stopped on ice by the addition of 2.5 mM Na_2EDTA and 0.1 mM phenylmethylsulfonyl fluoride, and the digest was centrifuged at 15,000 × g for 15 min. The soluble chromatin was layered onto 10-30% linear sucrose gradients containing 10 mM NaCl, 0.2 mM Na_2EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0, and centrifuged at 26,000 rpm for 18 h ( Beckman L3-50 centrifuge, SW 27 rotor) at 4 °C. The 15 S dinucleosome fractions were collected and used for anti-H5 antibody-binding studies.

H5 was removed from dinucleosomes according to the method of Ruiz-Carrillo et al. (16) using AG 50W-X2 (Bio-Rad) equilibrated with 0.35 m NaCl, 10 mM triethanolamine HCl, 0.4 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_2EDTA, pH 7.3. Dinucleosomes adjusted to 0.35 m NaCl were mixed with the resin (1:1, v/v) with gentle stirring for 2 h at room temperature. After centrifugation, the supernatant was dialyzed for 16 h against 10 mM triethanolamine, 0.4 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_2EDTA, pH 7.3, at 4 °C. The presence or absence of H5 in dinucleosomes was verified by SDS-gel electrophoresis. In order to quantitate the relative proportions of H5 in whole nuclei and dinucleosomes, 18% polyacrylamide gels were made in a Pasteur pipette, and the samples were eluted with buffer 50 mM NaCl, 1 mM Na_2EDTA, 50 mM Tricine, pH 8.0, and incubated at 40 °C for 2 min, followed by 20 min on ice. The mixture was fixed successively in 1% formaldehyde and 0.6% glutaraldehyde, on ice, for 15 min each (19).

Unbound antibodies were removed from the fixed dinucleosome/antibody mixture by Sepharose 2B chromatography (20) or sucrose gradient fractionation. A 1 ml bed volume Sepharose 2B column was made in a Pasteur pipette, and the samples were eluted with buffer containing 50 mM NaCl, 1 mM Na_2EDTA, 50 mM Tricine, pH 8.0. The void volume fractions containing dinucleosomes were collected and used for electron microscopy. In the case of sucrose gradient fractionation, the fixed dinucleosome/antibody mixture was centrifuged at 25,000 rpm for 17 h at 4 °C (SW 41 rotor) through a linear 10-30% sucrose gradient as described above. Dinucleosome peak fractions were collected and prepared for electron microscopy.

Electron Microscopy—A carbon film, deposited on freshly cleaved mica, was partially floated onto the dinucleosome solution (0.025 A260 units/ml). The film and mica were then withdrawn from the solution and completely floated off onto 2% aqueous uranyl acetate. A copper grid pretreated with 100% ethanol was used to pick up the carbon to form a double layer (21). The grid was blotted dry and examined with a Siemens 102 electron microscope.

RESULTS

Antibodies against H5 were IgG fractions isolated by ion exchange chromatography, and affinity purified fractions eluted from a Sepharose-H5 column. The globular peptide used for affinity chromatography gave a single band upon SDS-gel electrophoresis even when overloaded with sample. Both antibodies bound to H5 in a solid phase radioimmunoassay (Fig. 1) and did not cross-react with chick H1 in solid phase radioimmunoassay or with H1 bound to nitrocellulose paper in an electroblot (not shown). Antibodies against the globular peptide of H5 were isolated from the IgG fraction using a Sepharose globular peptide H5 affinity column; these also bound to H5 in a solid phase radioimmunoassay (Fig. 1).

The range of determinants for the total and affinity purified fractions was identified using an acetic acid digest of H5 electrophoretically blotted to nitrocellulose paper. Acetic acid cleaves the H5 molecule primarily at aspartic acid residues 65 and 99 (Fig. 2g). Under conditions of partial cleavage, five major peptides are produced (22), one of which (peptide 100-189) does not contain any of the globular region. Fig. 2, track a shows the peptide distribution on a stained 15% polyacrylamide-SDS gel. Four of the major peptides, labeled I, II, IV, and V, are visible; the origin of a fifth band, labeled III, is not known. Peptides 1-66 (V) and 1-99 (IV) were identified using a standard provided by Dr. Alice Mazen (Institut de Biologie Moleculaire et Cellulaire du Centre National de la Recherche Scientifique, Strasbourg, France) and characterized previously (22), while the other peptides were identified by electronphoretic mobility and lysine content. 2 Peptide 100-189 migrated more slowly than 1-99 even though it had a lower molecular weight. Track b of Fig. 2 shows the trypsin-derived globular peptide.

2 B. Neary and C. V. Mura, unpublished data.

PDF? paper in an electroblot (not shown). Antibodies against the globular peptide of H5 were isolated from the IgG fraction using a Sepharose globular peptide H5 affinity column; these also bound to H5 in a solid phase radioimmunoassay (Fig. 1).

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**Fig. 2.** Electrophoretic blotting of H5 histone peptides separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. -a, acetic acid digest-Coomassie blue stain (Roman numerals identify fragments shown in g); b, trypsin-derived globular peptide-Coomassie blue stain; c, acetic acid digest reacted with total anti-H5/RNA IgG; d, trypsin-derived globular peptide reacted with anti-H5/RNA IgG; e, acetic acid digest reacted with antiglobular peptide H5 antibody; f, trypsin-derived globular peptide reacted with antiglobular peptide antibody; g, diagram showing sites of trypsin and acetic acid cleavage of H5.

**Fig. 3.** 18% Polyacrylamide gel of chromatin fractions used for antibody binding. A, whole nuclear digest; B, purified dinucleosomes; C, dinucleosomes after removal of H1 and H5.

The IgG fraction of rabbit anti-H5/RNA bound to most of the major peptides (Fig. 2, track c), including those from both the NH2-terminal and COOH-terminal portions of the molecule. Binding to the trypsin-resistant globular peptide was

**Fig. 4 (top).** Sepharose 2B fractionation of dinucleosome/anti-H5 mixture. The upper portion of the micrograph contains the sample sandwiched with uranyl acetate stain between two layers of carbon. Present are antibody-dinucleosomes complexes (double arrowheads), free antibody (circled), and free dinucleosomes. The single carbon layer in the lower portion of the micrograph shows dinucleosomes (arrows), but antibody molecules are not visible.

**Fig. 5 (bottom).** Sucrose gradient fractionation of dinucleosome/anti-H5 mixture. Dinucleosome-antibody complexes (arrowheads) and free dinucleosomes are present. The increase in contrast compared with Fig. 4 is probably due to the sucrose present.
also observed (Fig. 2, track d). In contrast, the antiglobular peptide antibody bound to peptides 1-66 and 1-99, but not to 100-189 or 66-189 (Fig. 2, track e). As anticipated, this immunospecific fraction also bound to the trypsin-derived globular peptide (Fig. 2, track f). Since the globular portion of H5 extends to amino acid 99 (Fig. 2g), some binding of the antiglobular antibody to the 66-189 fragment might be expected. Since such binding was not observed, we conclude that most of the globular region antigenic determinants occur between residues 22 and 65. In a study of antibodies elicited by H5, which was not complexed with RNA, Mura et al. (37) found that the region 59-66 was a major antigenic determinant.

The group of anti H5-binding bands which migrated more slowly than whole H5 (Fig. 2, tracks c and e) was probably derived from H5, perhaps by an aggregation of the acetic acid peptides. The most compelling evidence for this is that antibody which was affinity purified using the globular peptide also bound to most of these bands (Fig. 2, track e). In any event, 90% of antibody binding (as determined by densitometer scans of the original autoradiographs) was to H5 and its characterized peptides.

Characterization of Chromatin— Dinucleosomes prepared as described contained intact H1, H5, and core histones as indicated by SDS-gel electrophoresis (Fig. 3), the level of H5 being 57% of that in whole nuclei. H1 and H5 were completely extracted from the dinucleosome preparation after treatment with the ion exchange resin AG 50W-X2 (Fig. 3).

The removal of unbound and excess antibodies from the fixed dinucleosome/antibody mixture by Sepharose 2B column fractionation was useful for small amounts of sample. However, the separation was not complete since free antibodies were present in the dinucleosome peak fraction (Fig. 4). Sucrose gradient fractionation, on the other hand, eliminated all the unbound antibodies (Fig. 5). At all ratios of antibody to chromatin which were tested (0.35–7.0, DNA/protein), the antibody-dinucleosome complexes co-migrated with unbound dinucleosomes in the gradient (not shown).

Immunoelectron Microscopy—Fixation of dinucleosomes and/or antibody-dinucleosome complexes prior to fractionation was found to be necessary to preserve the integrity of the dinucleosomes and to stabilize the attachment of the antibody to the dinucleosomes. During grid preparation (see "Materials and Methods"), unfixed dinucleosomes were partially unfolded, suggesting that the sandwich staining technique induces destabilization.

The use of the sandwich method (21) was found to be essential for the unambiguous visualization of antibody molecules. The uranyl acetate-positive stain commonly used for nucleosomes failed to reveal IgG molecules, while a conventional negative stain gave inconsistent results with both nucleosomes and antibodies. It should be noted that individual nucleosomes were increased in diameter by a factor of 1.4 in sandwich stained preparations as compared to single carbon layer methods. This is illustrated in Fig. 4, which shows specimen areas containing both single carbon film (bottom) and double carbon layers with the specimen and stain sandwiched between (top).

In control experiments using dinucleosome preparations without added IgG, 8.5% of the particles showed irregularities which would have been scored as bound antibodies. Another source of background was nonspecific binding of antibodies. This was assayed using normal rabbit IgG preparations and goat anti-rabbit IgG and by reacting anti-H5 preparations

### Table 1

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<tr>
<th>Source and type of antibody</th>
<th>H1/H5 present</th>
<th>Separation method</th>
<th>DNA/antibody ratio</th>
<th>Dinucleosomes scored</th>
<th>Dinucleosomes with bound IgG %</th>
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<td>Gradient</td>
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<td>358</td>
<td>8.9</td>
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<tr>
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<td>Gradient</td>
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<td>749</td>
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![Fig. 6. Examples of dinucleosome-anti-H5/RNA antibody complexes after Sepharose 2B fractionation illustrating the range of binding sites observed.](http://www.jbc.org/content/early/2018/01/17/jbc.M118.787702/F6.large.jpg)
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Fig. 7. As described in the legend to Fig. 6 except samples as from a sucrose gradient. The complex at the far right is a putative case in which one antibody is bound to two dinucleosomes.

Fig. 8. Histogram showing distribution of antibody-binding sites for anti-H5 (whole IgG fraction). The inset shows the site designations.

Fig. 9. Histogram showing distribution of binding sites using the affinity-purified antiglobular region antibody. The relative binding to position B is dramatically increased over that observed for the total antibody (Fig. 8).

with dinucleosomes from which H5 had been removed. In all three cases, close to 10% of dinucleosomes were scored as having bound antibodies (Table I). Thus, the binding and scoring techniques gave a combined “background” level of 10% antibody-dinucleosome complexes, of which about 8% was the result of dinucleosome irregularity, and the remainder, nonspecific binding.

All the anti-H5 antibodies tested showed above background binding to H5-containing nucleosomes (Table I), ranging from 23% for whole IgG to 6% for the two affinity purified fractions. Because it was not possible to reach saturation binding with the low concentration affinity purified preparations (Table I), the per cent binding values are of limited significance.

In spite of the similarity in size between antibody molecules and nucleosomes, it was possible in most instances to map the binding site in relation to the DNA linker attachment site. Figs. 6 and 7 show complexes of anti-H5/RNA IgG antibodies bound in a sequence of locations from opposite the linker DNA (left) to examples of attachment to the linker itself (right). Similar micrographs were obtained using the other classes of antibody (not shown). In order to analyze the binding patterns further, the nucleosome was partitioned into five areas, designated A–E (Fig. 8, inset), with A being the linker DNA, and E most distal to the linker site.

Figs. 8 and 9 show the distribution of antibody-binding sites for total anti-H5/RNA (Fig. 8) and affinity purified antiglobular region (Fig. 9). Both cases represent experiments in which sucrose gradient fractionation was used to remove free antibody, and background subtraction was performed separately for each area. (The distribution of background binding sites for the 9.2% of dinucleosomes reacted with rabbit normal IgG which showed apparent binding (Table I) was as follows: position A, 46%; B, 17%; C, 26%; D, 7%; and E, 3%.) Total anti-H5/RNA antibody bound most frequently to areas A and B, while regions distal to the linker showed progressively weaker binding (Fig. 8). All the classes of total anti-H5/RNA (Table I) showed a similar distribution of binding sites (data not shown). In contrast, the antiglobular region antibody showed a preference for site B on the nucleosome and close to the linker, while its binding to the linker DNA itself was markedly reduced (Fig. 9).

Most complexes contained one bound antibody/dinucleosome, although a few instances of two dinucleosomes bridged by a single antibody were found (Fig. 7). In sucrose gradient fractions, single antibodies attached to both mononucleosomes of a dinucleosome were common (Fig. 7).
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DISCUSSION

In order to use immunoelectron microscopy to map the position of histone H5 on nucleosomes, two principal conditions must be met. First, a fixed reference point on the nucleosome must be available for orienting antibody attachment sites, and second, both antibody and chromatin particle must be clearly visible in the electron microscope. Mononucleosomes have circular profiles in the electron microscope and thus, unlike ribosomal subunits (e.g. 21), cannot be used for mapping. Dinucleosomes, however, do have a reference point, since the entry/exit site of the linker DNA is fixed with respect to the histone octamer (23). Larger poly-nucleosomes are less satisfactory for mapping, except at low ionic strengths, nucleosome-nucleosome interactions cause compaction (4, 5, 24), which makes the antibody difficult to observe, and the antibodies themselves promote further compaction (25). The double carbon layer staining is one of the methods which enables both antibodies and chromatin particles to be resolved with satisfactory resolution and also has the advantage of minimizing drying stresses. Our results suggest that any distortion of the complexes tends to occur before the final staining. For example, the linker DNA is longer and more visible in Sepharose-purified material than after gradient separation (Fig. 6 versus Fig. 7). Nevertheless, the antibody binding distribution was very similar (not shown) and suggests that the location of H5 was not disturbed. In the future it may be advantageous to observe un-chromatin antibody complexes using scanning transmission electron microscopy (26).

The dinucleosomes used in the present study contained an average of one H5 molecule, rather than two, as would be expected from the stoichiometry in nuclei (18). A loss of H5 of this magnitude usually accompanies the production of dinucleosomes (27, 35), and is due to the partial removal of linker DNA-binding sites by the nuclease. This reduction in H5 accounts in part for the low proportion of antibody-antigen complexes observed (Table I). Of the two methods used for separating reactants, Sepharose 2B was less satisfactory since many free antibodies were present in the void volume (Fig. 4). On the other hand, sucrose gradient centrifugation removed all free antibody (Fig. 5), but provided no separation between free dinucleosomes and antibody-dinucleosome complexes. The absence of an increase in sedimentation velocity upon antibody binding in this case is presumably due to a balance between increased mass and increased Stokes radius for the complex.

Antibody-binding patterns were very similar for all the types of anti-H5 tested, with the preponderance of antibody molecules close to the linker DNA entry/exit site (Fig. 8). However, there were significant differences between the binding patterns of total anti-H5 and an affinity purified subfraction (Fig. 7), to study the accessibility of different portions of H2B (33), and to examine the distribution of H5 on chromatin fibers (25). Our use of anti-H5 antibodies to map the location of this histone on the nucleosome is a logical extension of these techniques. In the future, the availability of antibodies specific for the extended NH2- and COOH-terminal portions of H5 should allow a more precise localization of these regions.

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REFERENCES


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