Kornberg, R. D. (1977) *Annu. Rev. Biochem. 46*, 935.
Kumar, N. M., & Walker, I. O. (1980) *Nucleic Acids Res. 8*, 3535.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lin, W., & Kasamatsu, H. (1983) *Anal. Biochem. 128*, 302.
Mathiu, D. D. P., Mura, C. V., Frado, L. L., Woodcock, C. L. F., & Stollar, B. D. (1981) *J. Cell Biol. 91*, 135.
McGhee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115.
McGhee, J. D., Rau, D. C., Charney, E., & Felsenfeld, G. (1980) *Cell (Cambridge, Mass.) 22*, 87-96.
Mendelson, E., Smith, B. J., & Bustin, M. (1984) *Biochemistry* (following paper in this issue).
Muller, S., Himmelspach, K., & van Regenmortel, M. H. V. (1982) *EMBO J. 1*, 421.
Mura, C. V., & Stollar, B. D. (1981) *J. Biol. Chem. 256*, 9767.
Mura, C. V., Mazen, A., Neelin, J. M., Briand, G., Sautiere, P., & Champagne, M. (1980) *Eur. J. Biochem. 108*, 613.
Neelin, J. M., Callagan, P. X., Lamle, D. C., & Murray, K. (1964) *Can. J. Biochem. 42*, 1743.
Panyim, S., & Chalkley, R. (1969) *Biochem. Biophys. Res. Commun. 37*, 1042.
Pehrson, J. R., & Cole, R. D. (1980) *Nature (London) 285*, 43-44.
Pehrson, J. R., & Cole, R. D. (1981) *Biochemistry 20*, 2298-2301.
Renz, M., Nehls, P., & Hozier, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 47*, 1879-1883.
Romani, M., Vidali, G., Tahourdin, C. S. M., & Bustin, M. (1980) *J. Biol. Chem. 252*, 468-474.
Simpson, R. T. (1978) *Biochemistry 17*, 5524-5531.
Smith, B. J., Walker, J. M., & Johns, E. W. (1980) *FEBS Lett. 112*, 42-44.
Thoma, F., Losa, R., & Koller, T. (1983) *J. Mol. Biol. 167*, 619-640.
Thomas, J., & Rees, C. (1983) *Eur. J. Biochem. 136*, 109-115.

Mapping the Binding of Monoclonal Antibodies to Histone H5†

Ella Mendelson, B. J. Smith,‡ and Michael Bustin*

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).

† From the Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received November 28, 1983.*
‡ Present address: Department of Molecular Biology, Chester Beatty Research Institute, London, SW3 6JB, England.

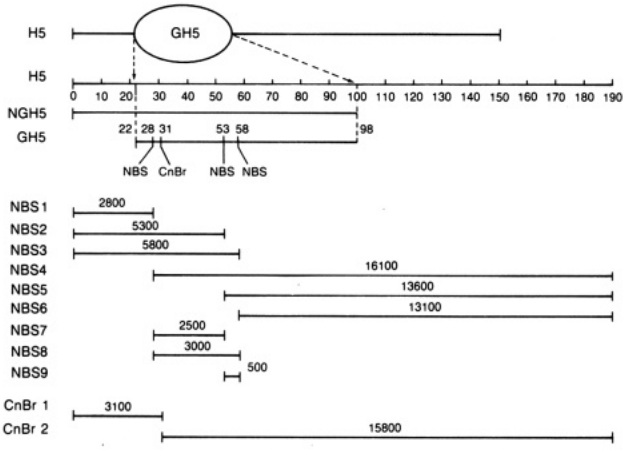

FIGURE 1: Peptides generated from H5. Histone H5 has a globular region between residues 22 and 98. The points of cleavage of H5 by *N*-bromosuccinimide and cyanogen bromide are indicated by the residue number and NBS or CNBr, respectively. The relative positions of the peptides and their approximate molecular weights are also indicated in the diagram.

Chemical Cleavage of H5. *N*-Bromosuccinimide (NBS)[1] cleavage of histone H5 was performed as described previously (Ramachandran & Witkop, 1967; Bustin & Cole, 1969). To obtain a mixture of all the possible peptides generated by controlled *N*-bromosuccinimide digestion, aliquots were sampled during the reaction and examined by electrophoresis in polyacrylamide gels. *N*-Bromosuccinimide dissolved in 50% acetic acid was added to H5 in 50% acetic acid at a molar ratio of 20:1 at room temperature. The reaction was performed in an Eppendorf tube in a total volume of 100 μL. After 1.5 h of reaction, 1000 μL of H_2O was added to the tubes, the mixture was dried in a Speed-Vac centrifuge, and the peptides were resuspended in H_2O at 2 mg/mL.

Cyanogen bromide treated H5 which contained a mixture consisting of uncleaved H5 and the two peptides was generated by partial cleavage of the molecule at methionine-31 (Smith et al., 1980).

Proteolytic Digestions of H5. Bovine trypsin (Worthington) dissolved in 1 mM HCl was added to H5 dissolved at 1.0 mg/mL in 0.1 M NaCl, 0.01 M sodium phosphate buffer, pH 7, and 50 mM $CaCl_2$ at a substrate to enzyme ratio of 1250:1. At various times of reactions, the digestion was stopped by adding soybean trypsin inhibitor at a 2-fold molar excess over trypsin.

For pepsin digestion, the enzyme (Worthington) was dissolved in 0.01 M HCl and added to histone H5 at pH 4 (acetate buffer) at a substrate to enzyme weight ratio of 2000:1. After various times of reaction, the digestion was stopped by adding 0.2 M NaOH until the pH was 7.0.

Electrophoresis and Immunoblotting. The proteins and peptides were electrophoresed in 18% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Laemmli, 1970). Transfer of the polypeptides from the gels to nitrocellulose sheets, subsequent treatment with antibody, and autoradiographic detection were performed as described in the preceding paper (Mendelson & Bustin, 1984). The intensity of the bands on the autoradiogram was quantitated by densitometric scanning in a Beckman DU-8 spectrophotometer.

Results

To map the location of the epitopes along the polypeptide chain of H5, a series of peptides was generated by several methods and the binding of the various monoclonals to the peptides determined by immunoblotting.

[1] Abbreviations: NBS, *N*-bromosuccinimide; CNBr, cyanogen bromide; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; HMG, high-mobility group.

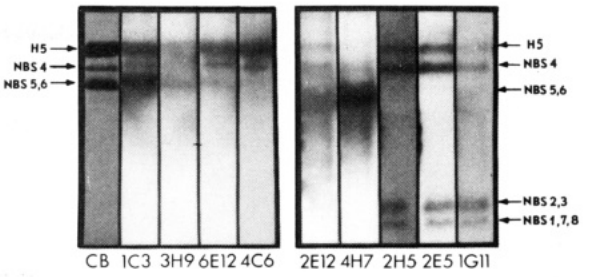

FIGURE 2: Interaction of monoclonal antibodies with peptides generated by *N*-bromosuccinimide cleavage of H5. The figure presents the autoradiograms of the nitrocellulose sheets after reaction with the monoclonal antibody indicated at the bottom of each lane. CB indicates the Coomassie blue stain of the transferred gel. The locations of uncleaved H5 and of the various NBS peptides are indicated in the margins of the figure.

The main structural features of histone H5 which are pertinent to the present study are diagrammed in Figure 1. Chicken erythrocyte histone H5 contains 189 amino acids (Briand et al., 1980). A central trypsin-resistant globular region (denoted as GH5) ranging from amino acid 22 to amino acid 98 can be isolated by controlled trypsin digestion (Aviles et al., 1978). The trypsin digest contains a peptide, denoted as NGH5, which migrates somewhat slower than GH5 and contains the 98 N-terminal amino acids. Cleavage of the protein with cyanogen bromide at the single methionine in position 31 bisects the molecule, yielding peptides CNBr1 and CNBr2 with approximate molecular weights of 3100 and 15800, respectively. partial cleavage with *N*-bromosuccinimide at tyrosine residues 28, 53, and 58 yields a set of nine peptides with the approximate molecular weights indicated. (The molecular weights of the peptides were estimated by multiplying the number of amino acid residues by 100.) Most peptides can be resolved by their mobility on polyacrylamide gels. Thus, peptides GH5 and NGH5 electrophorese as a close doublet (Allan et al., 1982). Peptides CNBr1 and CNBr2 are easily separated on 18% polyacrylamide gels. Partial *N*-bromosuccinimide cleavage of H5 yields four major bands, one containing peptide NBS4, one containing peptides NBS5 and NBS6, one containing NBS2 and NBS3, and one containing peptides NBS1, NBS7, and NBS8 (see data below).

Binding of Monoclonal Antibodies to NBS Peptides. The conditions under which H5 is partially cleaved to produce a series of peptides were determined in a series of experiments in which the ratio of *N*-bromosuccinimide to H5 and the time of reaction were systematically varied. Reaction for 90 min at room temperature at a molar ratio of reagent to H5 of 20 yielded a mixture of peptides, suggesting that all possible peptides have been generated. The reaction mixture was electrophoresed on 18% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Staining of the gels with Coomassie blue visualized residual uncleaved H5, peptide NBS4, and a band which contained both peptides NBS5 and NBS6. The small molecular weight peptides which were lost during destaining were detected by immunoblotting. The peptides present in the gel were transferred electrophoretically to nitrocellulose sheets which were subsequently processed for immunoblotting with the various monoclonal antibodies. The results presented in Figure 2 indicate that the various antibodies reacted with different sets of peptides. Thus, antibodies 2E5, 1G11, and 2H5 reacted with intact H5 and with bands containing peptides NBS4, NBS2,3, and NBS1,7,8 but not with NBS5,6. Therefore, the antigenic sites of these antibodies must reside between residues 28 and 53. Monoclonals 4H7 and 1C3 gave

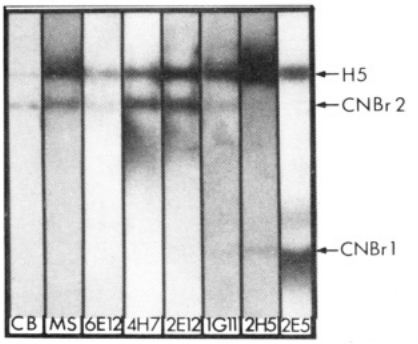

FIGURE 3: Interaction of monoclonal antibodies with peptides generated by bisecting histone H5 with cyanogen bromide. The figure depicts autoradiograms of the nitrocellulose sheets after reaction with the monoclonal antibody indicated at the bottom of the various lanes. CB indicates the Coomassie blue stain of the transferred gel. MS indicates mouse serum.

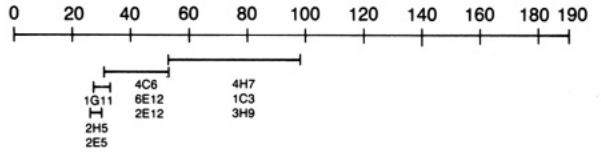

FIGURE 4: Mapping epitopes in histone H5. The binding sites of the various monoclonals in the schematically linearized H5 molecule are indicated.

strong positive reactions with NBS5,6 and weak reactions with NBS4. They did not react with the smaller NBS peptides. Previous studies (Mendelson & Bustin, 1984) indicated that they react with GH5, which spans residues 22–98. Therefore, these two monoclonals react with epitopes located between residues 58 and 98. Monoclonals 4C6 and 6E12 essentially react only with NBS4 and H5. This epitope is therefore located between residues 28 and 53. These two antibodies did not react with peptides NBS2,3 and NBS1,7,8. Possibly the conformation of the epitope may depend on the presence of the C-terminal region of GH5, or, alternatively, the binding of the small peptides to nitrocellulose sterically hinders the antigenic site. Monoclonal 2E12 gave a faint pattern resembling that obtained with 6E12 and 4C6. Monoclonal 3H9 gave a weak signal which qualitatively resembles the pattern obtained with 1C3. This antibody may bind to the same general region as 1C3; however, the epitope may be denatured during the NBS cleavage, or it may be sterically hindered on the nitrocellulose sheet.

Binding to Cyanogen Bromide Peptides. The autoradiographs obtained upon immunoblotting of the antibodies with the two peptides generated by cyanogen bromide are presented in Figure 3. Monoclonals 2H5, 2E5, and 1G11 gave a positive reaction with the small peptide CNBr1, which spans amino acid residues 1–31. This observation, taken together with the binding pattern of these three monoclonals to the peptides generated by NBS, pinpoints the epitope to a narrow region encompassing residues 28–31. In agreement with the results obtained with the NBS peptides, antibodies 6E12, 4H7, and 2E12 bound to the large CNBr2 peptide.

Further Characterization of the Monoclonal Antibodies. The diagram presented in Figure 4 localized the nine antibodies studied. The binding sites of the antibodies map into three main clusters. The epitope for antibodies 1G11, 2H5, and 2E5 can be pinpointed to a narrow region spanning residues 28–31. Monoclonals 4C6, 6E12, and 2E12 bind to an epitope present between amino acids 28 and 53, while monoclonals 4H7, 1C3, and 3H9 map in a region encompassed by amino acid residues 53–98. The proximity of the binding site for monoclonals 2H5

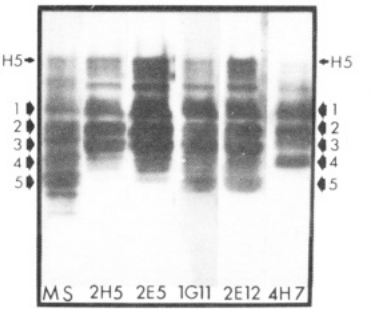

FIGURE 5: Interaction of monoclonal antibodies with peptides generated from H5 by controlled digestion with trypsin. The peptides were transferred to nitrocellulose sheets which were reacted with the monoclonals indicated at the bottom of the various lanes and autoradiographed. Lane MS was reacted with mouse anti-H5 serum. Numbers 1–5 at the margins of the figure are used for identification of various regions in the autoradiogram (see text).

and 2E5 is further verified by their pattern of interaction with a partial trypsin digest of H5. The autoradiogram presented in Figure 5 indicates that monoclonals 2H5 and 2E5 bind to identical tryptic peptides. Furthermore, ELISA experiments in which the two antibodies were added simultaneously to the antigen clearly demonstrate that the mix gives a pattern indicative of competitive binding.

In the competitive ELISA experiments, the binding of two antibodies added simultaneously is compared to the binding of each antibody when added by itself. Note that at low antibody concentrations, when the epitopes are not saturated, the mix gives a reaction which is higher than that given by each member of the mix added separately. At saturation, the reaction of the mix approximates the average reaction of each of the sera. In these experiments, the higher OD_{405} values obtained with monoclonals 2H5 and 2E12 as compared to 2E5 are due to the fact that 2H5 and 2E12 are IgM antibodies and presumably can bind more alkaline phosphatase labeled anti-mouse Ig than antibody 2E5, which is IgG. The competitive binding experiments also indicate competition between monoclonals 1G11 and 2H5 and 2E5 [see Figure 6C here and Figure 6 of the previous paper (Mendelson & Bustin, 1984)]. However, the exact epitope of 1G11 is different from that of 2E5 and 2H5 as evidenced by the binding of this monoclonal to a trypsin digest of H5 (Figure 5) or to the isolated GH5 region (Figure 7). In Figure 5, it can be seen that 1G11 reacts with peptides migrating in region 5. Monoclonals 2E5 and 2H5 do not react with this region. Immunoblots with purified GH5 peptide (Figure 7) further support the differences between 1G11 and 2H5/2E5. This peptide is contaminated with small amounts of NGH5 which are not detectable by Coomassie blue staining but can be detected by the more sensitive immunoblotting techniques. Clearly, 1G11 does not bind to GH5 but to NGH5, while 2E5 and 2H5 bind mostly to GH5. Obviously, since GH5 is contained within NGH5, monoclonal 1G11 was expected to bind to both peptides. The failure to bind to GH5 could represent a situation where the presence of the N-terminal tail influences the structure of the globular GH5 region (Barbero et al., 1982) so that the 1G11 epitope is stabilized. Alternatively, the GH5 peptide interacts with the nitrocellulose in a way which causes steric hindrance to antibody binding.

The epitopes for monoclonals 4C6, 6E12, and 2E12 which map in the peptide region 28–53 are distinct not only from the monoclonals which bind to region 28–31 but also from each other. The difference between the two groups of monoclonals is established by their pattern of interaction with CNBr- and NBS-generated peptides and by competitive ELISA experi-

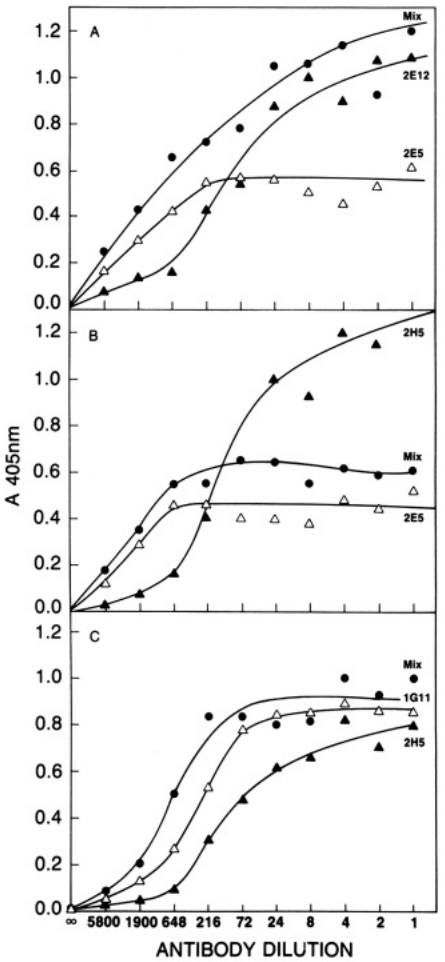

FIGURE 6: Competitive ELISA inhibition experiments indicating whether epitopes are shared or closely spaced. The antibodies were added to H5 and immobilized on microtiter plates, either as individual components or in pairs. The antibody bound was measured by ELISA. The A_{405} developed 1 h after addition of substrate at room temperature is shown. (A) Antibodies 2E5 and 2E12 and a mixture. Note that the mixture gave higher values than each of the individual antibodies, indicating lack of competition. (B) Antibodies 2H5 and 2E5 and a mix of both. Note that the mix gives absorbancy values intermediate between the two individual antibodies, indicating that the antibody bound to the same epitope or that the epitope is closely spaced so as to cause steric hindrance. (C) Antibodies 1G11 and 2H5 and a mix. The values obtained by the mix indicate close proximity of the epitopes.

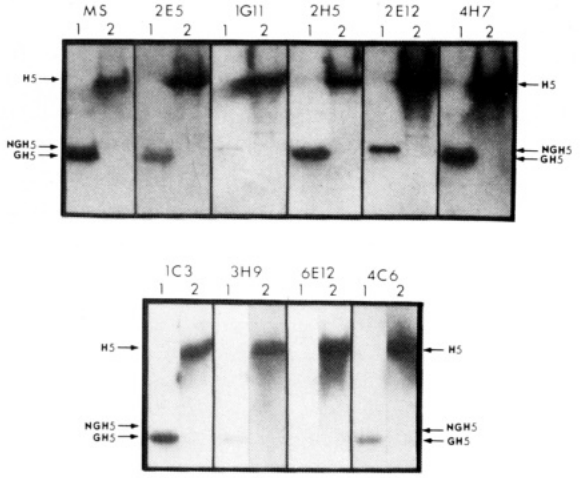

FIGURE 7: Binding of monoclonals to peptides GH5, NGH5, and intact H5. Intact H5 or purified GH5 containing traces of NGH5 was transferred from polyacrylamide gels to nitrocellulose sheets which were reacted with the monoclonal antibodies indicated on top of the various panels and autoradiographed. Lane 1 contains GH5 and NGH5; lane 2 contains H5. MS, mouse anti-H5 serum.

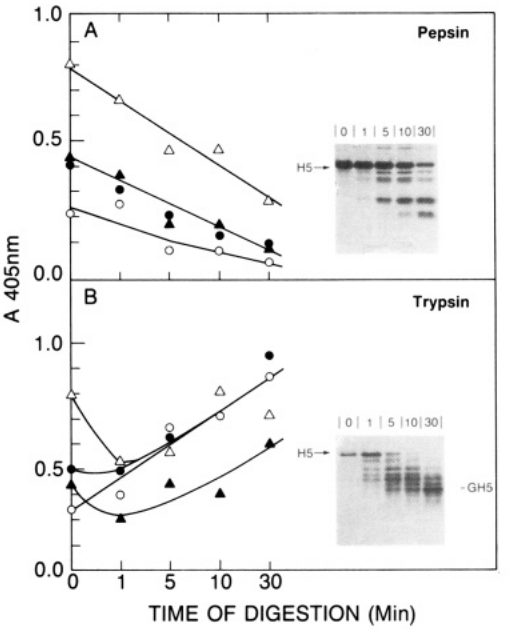

FIGURE 8: Effect of proteolytic digestion of H5 on the stability of epitopes. H5 was digested with either pepsin (A) or trypsin (B). At various times of digestion, aliquots were removed, and the survival of various epitopes was tested by ELISA with various monoclonal antibodies. The pattern of peptides produced and the amount of undigested H5 present after various times of digestion can be estimated from the polyacrylamide gels presented as insets in panels A and B. (▲) 3H9; (△) 1C3; (○) 4C6; (●) 6E12.

ments. Interestingly, monoclonals 1G11 and 2E12 share some common characteristics. The patterns of binding to a trypsin digest of the two monoclonals are indistinguishable (see Figure 5), as are their interactions with NGH5. Monoclonal 2E12 gave a very strong signal with NGH5 and no detectable signal with GH5 (Figure 7). Careful examination of the CNBr binding data in Figure 3 reveals that monoclonal 1G11 gives a weak signal with CNBr2. It seems, therefore, that epitope 1G11 partially overlaps both epitopes 2H5/2E5 and 2E12. It is noteworthy that monoclonal 6E12 completely failed to give a signal with either GH5 or NGH5. Apparently, the epitopes in the region centering around amino acid 31 bind to nitrocellulose in a manner which causes steric hindrance to this antibody binding.

The third group of monoclonals, antibodies 4H7, 1C3, and 3H9, binds to epitopes in the peptide region 53–98. The pattern of binding to tryptic peptides of monoclonal 4H7 is different from those of the other two epitope clusters. The autoradiogram in Figure 5 shows that this monoclonal does not bind to peptides in region 3 but binds to peptides in region 4. The three monoclonals belonging to this group bind to GH5 which was immobilized on nitrocellulose sheets. Competitive ELISA tests indicate that the three epitopes are spatially separated so that no steric hindrance is observed.

In an effort to generate additional peptides, to further identify the location of the epitopes, H5 was digested with either pepsin or trypsin. At various times of digestion, aliquots were tested by ELISA for the survival of the epitope for various monoclonal antibodies. The results, presented in Figure 8, indicate that pepsin digestion destroyed all the epitopes tested. A comparison of the residual antigenic activity with the amount of intact H5 left in the digest (see polyacrylamide gels in the inset of Figure 8A) suggests that the residual antigenic activity is due to the presence of intact H5 and that all the epitopes tested were sensitive to pepsin digestion. In contrast, trypsin digestion of H5 did not diminish the binding of the monoclonal to the digest. In fact, in some cases, the digests

Table I: Interaction[a] of Monoclonal Antibodies with Various Histone H5 Fragments

	antibody										
peptide	1C3	6E12	4C6	3H9	2E12	4H7	2E5	1G11	2H5	αH5 mouse serum	αH5-RNA mouse serum
NBS2,3	–	–	–	–	–	–	+	+	+	+	+–
NBS4	+	+	+	+–	+	+–	+	+	+	+	+–
NBS5,6	++	–	–	+	+–[b]	++	–	–	–	+	+–
NBS1,7,8	–	–	–	–	–	–	+	+	+	–	–
CNBr1	NT[c]	–	NT	NT	–	–	++	+	+	NT	–
CNBr2	NT	+	NT	NT	+	+	–	+–	–	NT	+
GH5	++	–	+	+	–	+	+	–	+	+	+
NGH5	–	–	–	–	++	+–	+	+	–	–	+

[a] Interaction determined by immunoblotting. [b] +– = weakly positive. [c] NT = not tested.

bound the monoclonals more efficiently. The increase in the binding could be correlated with the disappearance of intact H5 and the generation of GH5 molecules.

Discussion

Table I summarizes the reactivity of the various monoclonal antibodies with the different peptides generated from H5. All the monoclonal antibodies reacted with GH5 [monoclonal 6E12 reacted with GH5 in the ELISA assay; see Mendelson & Bustin (1984)]. Thus, all monoclonal antibodies bind to peptides 22–98. 2E5 and 2H5 bind to all the NBS peptides except NBS5,6, indicating that the epitope does not extend beyond residue 53. The binding of the antibodies to CNBr1 but not to CNBr2 pinpointed the main binding site to the region 28–31. Competitive ELISA experiments and the pattern of binding to tryptic peptides further verified the fact that these two antibodies have identical or very closely spaced epitopes. Monoclonal 1G11 binds to the same NBS peptides as 2E5/2H5. However, its binding to tryptic peptides and its weak interaction with CNBr2 suggest that its epitope partially overlaps the epitope of monoclonal 2E12. The epitopes for monoclonals 2E12, 6E12, and 4C6 are located within a stretch of 22 amino acids between residues 31 and 53. The main evidence for their location is their binding to CNBr2 and NBS4 but not to NBS5,6. Monoclonals 4H7, 1C3, and 3H9 bind significantly better to NBS5,6 than to NBS4, indicating that their epitopes are located within a stretch of 45 amino acids from residue 53 to residue 98.

The mapping of the epitopes in the H5 molecule is based on the immunoblotting technique. This approach is essentially appropriate since it not only detects but also unequivocally identifies the antigen. Immunoblotting minimizes the possibility that the monoclonal is directed against a minor contaminant which is not detected when the purity of the antigen is examined, most commonly by polyacrylamide gels. A case in point are monoclonals 2E12 and 1G11. ELISA experiments indicate that they bind to a preparation of pure GH5, yet the sensitive immunoblotting technique indicated that the antibody actually bound to NGH5, which was a minor contaminant in the GH5 preparation. The main pitfall of the immunoblotting technique is that in some cases antibodies which interact with an antigen in ELISA or in solution fail to bind to the antigen which is immobilized on nitrocellulose. This binding raises the possibility that the antigen binds to the solid support in an oriented, nonrandom fashion. It is noteworthy that such a situation will not give a false positive signal and therefore will not lead to mistaken identification of an antigen.

The number of monoclonal antibodies tested does not allow statistically significant conclusions regarding the imunogenicity of the various regions of H5. Yet it is noteworthy that all the monoclonals produced were directed toward the GH5 region. Most of the antibodies present in polyclonal sera are also directed against this region (Allan et al., 1982). Within the GH5 region, three monoclonals localized within a narrow region close to the N terminus of the globular region. Interestingly, these monoclonals were obtained by immunizing with H5·RNA complexes rather than with free H5 (Mendelson & Bustin, 1984). Mice immunized with free H5 tend to give monoclonals which react with epitopes located centrally or toward the C terminus of the globular region. The situation is similar with polyclonal antibodies since Mura et al. (1980) identified a region around residues 94–96 as a strong antigen and around residues 59–65 as a moderate antigen in sera from guinea pigs immunized with H5.

In chromatin, the proteins are organized in a dynamic macromolecular complex whose morphological appearance and metabolic activity change during the life cycle of a cell. The advantage of using serological techniques to study the organization of specific chromosomal components at various stages of chromatin organization has already been pointed out (Bustin, 1979). Definition of the antigenic sites of nucleoproteins would further enhance the usefulness of the immunochemical approach to chromatin structure. Studies with various histone fractions and HMG proteins allocated the antigenic activity to a number of large peptide fragments. In an effort to get more precise immunochemical reagents, Muller et al. (1982) elicited polyclonal antibodies against a chemically synthesized hexapeptide of histone H3. An obvious approach toward obtaining antibodies with defined, narrow specificity is the monoclonal antibody technique. However, to fully realize the potential of this approach, it is necessary to define and map the location of the epitope in the antigen. In the present study, we have mapped the location of the epitopes for nine monoclonals with sufficient precision to facilitate studies on the interaction of particular segments of histones H5 and H1° with other chromosomal constituents.

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References

Allan, J., Hartman, P. G., Crane-Robinson, C., & Aviles, F. X. (1980) *Nature (London) 288*, 675–679.

Allan, J., Smith, B. J., Dunn, B., & Bustin, M. (1982) *J. Biol. Chem. 257*, 10533–10535.

Aviles, F. J., Chapman, G. E., Kneale, G. G., Crane-Robinson, C., & Bradbury, E. M. (1978) *Eur. J. Biochem. 88*, 363–371.

Barbero, J. L., & Montero, F. (1982) *Biochem. Biophys. Res. Commun. 107*, 842–847.

Bradbury, E. M., Carpenter, B. G., & Rattle, H. W. E. (1973) *Nature (London) 241*, 123–126.
Briand, G., Kmiecik, D., Sautiere, P., Wouters, D., Borie-Loy, O., Bigerte, G., Mazen, A., & Champagne, M. (1980) *FEBS Lett. 112*, 147–151.
Bustin, M. (1979) *Curr. Top. Microbiol. Immunol. 88*, 105–142.
Bustin, M., & Cole, R. D. (1969) *J. Biol. Chem. 244*, 5291–5294.
Butler, P. J. G., & Thomas, J. O. (1981) *J. Mol. Biol. 140*, 505–529.
Cary, P. D., Hines, M., Bradbury, E. M., Smith, B. J., & Johns, E. W. (1981) *Eur. J. Biochem. 120*, 371–377.
Chabanas, A., Lawrence, J. J., Humbert, J., & Eisen, H. (1983) *EMBO J. 2*, 833–837.
Kumar, N. M., & Walker, I. O. (1980) *Nucleic Acids Res. 8*, 3535–3551.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
McGhee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115–1156.
Mendelson, E., & Bustin, M. (1984) *Biochemistry* (preceding paper in this issue).
Muller, S., Himmelspach, K., & Van Regenmortel M. H. V. (1982) *EMBO J. 1*, 421–425.
Mura, C. V., Mazen, A., Neelin, J. M., Briand, G., Sautiere, P., & Champagne, M. (1980) *Eur. J. Biochem. 108*, 613–620.
Neelin, J. M., Callaghan, P. X., Lamb, D. C., & Murray, K. (1964) *Can. J. Biochem. 42*, 1743–1752.
Pehrson, J., & Cole, R. D. (1980) *Nature (London) 285*, 43–44.
Ramachandran, L. K., & Witkop, B. (1967) *Methods Enzymol. 13*, 283.
Renz, M., Nehls, P., & Hozier, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1879–1883.
Seligy, V. L., Adams, G. H. M., & Neelin, J. M. (1973) in *Biochemistry of Gene Expression in Higher Organism* (Pollah, J., & Lee, J. W., Eds.) pp 177–190, Australia and New Zealand Book Co., TTY, Sydney.
Simpson, R. T. (1978) *Biochemistry 17*, 5524–5531.
Smith, B. J., Walker, J. M., & Johns, E. W. (1980) *FEBS Lett. 112*, 42–44.
Sung, M. T., & Freedlender, E. F. (1978) *Biochemistry 17*, 1804–1878.
Thoma, F., Koller, T., & Klug, A. (1979) *J. Cell Biol. 93*, 403–427.
Thomas, J. O., & Rees, C. (1983) *Eur. J. Biochem. 134*, 109–115.

Zinc Potentiation of Androgen Receptor Binding to Nuclei in Vitro†

Douglas S. Colvard and Elizabeth M. Wilson*

ABSTRACT: Zn^{2+} potentiates binding of the 4.5S [^{3}H]dihydrotestosterone-receptor complex to isolated rat prostate Dunning tumor nuclei in vitro when assayed in the presence of 300 μM $ZnCl_2$, 3 mM $MgCl_2$, 0.25 M sucrose, 5 mM mercaptoethanol, 0.15 M KCl, and 50 mM tris(hydroxymethyl)aminomethane, pH 7.5. In the presence of 5 mM mercaptoethanol, the concentration of 50 μM total Zn^{2+} required to promote half-maximal receptor binding to nuclei corresponds to a free Zn^{2+} concentration of 50 nM. The receptor–nuclear interaction appears to be selective for Zn^{2+}; other divalent cations when added at a concentration of 1 mM to a buffer containing 5 mM mercaptoethanol are less effective (Ni^{2+}) or have essentially no effect (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+}). Zn^{2+} does not alter the sedimentation rate of the 4.5S [^{3}H]dihydrotestosterone receptor in the presence of mercaptoethanol; however, in the absence of mercaptoethanol, Zn^{2+} causes the receptor to aggregate. Zn^{2+}-dependent nuclear binding of the 4.5S [^{3}H]dihydrotestosterone receptor is saturable at 1.4×10^{-13} mol of receptor sites/mg of DNA, corresponding to approximately 1150 sites/nucleus. In the presence of excess nuclei, up to 60% of added receptor is nuclear bound. An apparent binding constant for the receptor–nuclear interaction of 10^{13} M^{-1} was approximated. Pyridoxal 5′-phosphate ($\leq$10 mM), but not 0.4 M KCl, inhibits Zn^{2+}-dependent nuclear binding of the [^{3}H]dihydrotestosterone receptor. Up to 66% of nuclear-bound receptor can be extracted in buffer containing 3 mM ethylenediaminetetraacetic acid plus either 0.4 M KCl or 10 mM pyridoxal 5′-phosphate. Nuclear receptor extracted in buffer containing the protease inhibitor diisopropyl fluorophosphate (2 mM) sediments at 4.5 S on sucrose gradients, but the receptor sediments at 3 S when extracted without the inhibitor. Zn^{2+}-dependent nuclear binding of the [^{3}H]dihydrotestosterone receptor is temperature dependent, with association rate constants (k_a) at 0, 15, and 25 °C of 4.0×10^5, 2.8×10^6, and 9.7×10^6 M^{-1} min^{-1}, respectively. The activation energy of binding is 21 kcal/mol.

Androgens are believed to be concentrated in target cell nuclei through an unknown mechanism involving the androgen receptor (Rennie & Bruchovsky, 1973). Attempts to mimic this process of nuclear retention of steroid receptors in vitro have led to the realization that an alteration in the receptor occurs that is likely initiated by the binding of hormone. The terms "transformation" and "activation" have been adopted to encompass this poorly understood process whereby the steroid-receptor complex is converted to a nuclear or DNA binding form (Alger & Milgrom, 1976; Kalimi et al., 1975; Higgins et al., 1973; Buller et al., 1975a,b). In vitro transformation of certain steroid receptors can be induced by heat (Higgins et al., 1973; Buller et al., 1975a,b), dilution (Higgins et al., 1973), $(NH_4)_2SO_4$ (Buller et al., 1975a,b), or ATP (Moudgil et al., 1981).

Studies on androgen receptor binding to isolated nuclei have been complicated by the low concentration of receptor, the susceptibility of the receptor to proteolytic cleavage (Wilson

† From the Departments of Pediatrics and Biochemistry and the Laboratories for Reproductive Biology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514. *Received November 1, 1983.* This work was supported by U.S. Public Health Service Research Grants HD16910 and HD04466 and by American Cancer Society Institutional Grant IN-15-V.

* Address correspondence to this author at the Department of Pediatrics, University of North Carolina School of Medicine.

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