Antigenic Determinants of High Mobility Group Chromosomal Proteins 1 and 2†

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ABSTRACT: The antigenic determinants of nonhistone high mobility group chromosomal proteins 1 (HMG-1) and 2 (HMG-2) were studied with rabbit antisera elicited against HMG-1 and against HMG-2 and monoclonal antibodies elicited by HMG-1. The monoclonal antibodies did not distinguish between the two proteins, suggesting that they have specificity toward a shared determinant. Whereas anti-HMG-1 did not, anti-HMG-2 did distinguish between the proteins, suggesting that the anti-HMG-2 serum contains antibodies against peptides which differ between the proteins. Peptides were generated from HMG-1 and HMG-2 by controlled digestion with trypsin and pepsin. Analysis of the digests by ELISA and by sodium dodecyl sulfate electrophoresis followed by diazobenzyloxymethyl transfer, antibody binding and autoradiography revealed that most of the antibodies are against sequential determinants some of which are smaller than 3000 in molecular weight.

High mobility group chromosomal proteins 1 (HMG-1)[1] and 2 (HMG-2) are among the few nonhistones which have been purified to homogeneity and whose primary sequence is known. These two proteins are very similar in many respects such as primary structure (Walker et al., 1980), molecular weight (Baker et al., 1976; Goodwin et al., 1975), ubiquitous distribution among several kingdoms (Spiker et al., 1978; Sterner et al., 1978; Romani et al., 1979), ability to bind to DNA (Shooter et al., 1974; Javaherian et al., 1978), localization to the linker regions between nucleosomes (Levy et al., 1977; Vidali et al., 1977), and serological properties (Romani et al., 1979; Vidali et al., 1979). The cellular role of the proteins is presently not known. Generally it is accepted that these two proteins have a structural rather than regulatory role (Goodwin & Johns, 1978). In some cases, however, an increase in the relative amount of HMG-2 in rapidly replicating tissues has been observed (Seyedin & Kistler, 1979).

Elucidation of the role of various chromosomal proteins, in maintaining the structure and regulating the function of chromatin and chromosomes, is hampered by the fact that most proteins do not have a function which is easy to assay. In lieu of functional assays specific antibodies can serve as tools to identify, quantitate, localize, and study the in vivo function or defined components in chromatin and chromosomes (Bustin, 1979). Antibodies elicited by HMG-1 can react with HMG-2 and bind to both interphase chromatin (Bustin et al., 1977) and polytene chromosomes (Kurth & Bustin, 1981). Immunofluorescence techniques used to study the cellular localization of the proteins yielded conflicting results: the proteins have been detected both in the nucleus and in the cytoplasm of various cells (Bustin & Neihart, 1979; Smith et al., 1978; Bhullar et al., 1981).

The ability of antibodies to bind to intracellular components depends on the accessibility of the antigenic sites. Insights into the nature of the antigenic sites of chromosomal proteins HMG-1 and HMG-2 will help evaluate the reliability of various experiments in which antibodies are used. In the present paper monoclonal and polyclonal antibodies to HMG-1 and HMG-2 are used in conjunction with protease digestions to gain a better understanding of the antigenic sites of these chromosomal proteins.

Materials and Methods

Proteins and Polyclonal Antibodies. The purity of chromosomal proteins HMG-1 and HMG-2 used in this study has been previously established (Bustin et al., 1977). Antibodies to chromosomal protein HMG-2 were elicited by injecting four rabbits at multiple intradermal sites as described for HMG-1 (Bustin et al., 1977). Cross-reactivity with HMG-1 was reduced by passing the sera on Sepharose columns to which HMG-1 was bound.

Monoclonal Antibodies. The basic procedure of Kohler & Milstein (1977) was followed. Six Balb/C mice were injected subcutaneously each with 50 μg of HMG-1 in 66% complete Freund's adjuvant. A booster of 50 μg of HMG-1 in PBS was administered 14 days after immunization. Seven days after the booster injection the sera from the mice were checked for anti-HMG-1 activity. The mice which elicited antibodies were given an additional booster, and 4 days later their spleens were removed. For fusion, 1×10^7 myeloma NS-1 cells were fused with 1×10^8 immune spleen cells in 45% poly(ethylene glycol). After fusion the cells were seeded into 96 well microtiter plates. The supernatants were tested for production of antibody to HMG-1 by a solid phase radioimmunoassay using ^{125}I-labeled Ig anti-mouse Ig produced in sheep. Hybrid cells from positive wells were cloned 2 times at limiting dilutions. Ascites fluid containing anti-HMG-1 activity was produced by injection of 5×10^6 cells into the peritoneal cavity of Balb/C mice 1 day after intraperitoneal injection of 0.5 mL of 2,6,10,14-tetramethylpentadecane.

Immune Assays. Ouchterlony immunodiffusion was performed at pH 8.0 in plates obtained from Cappel Laboratories. Solid phase radioimmunoassay of rabbit antisera was performed as described previously (Romani et al., 1980). Bound mouse IgM was detected by using IgG from rabbit anti-mouse IgM (Miles) followed by ^{125}I-labeled sheep anti-rabbit Ig (New England Nuclear). Enzyme-linked immunoassay of rabbit antibodies was done with alkaline phosphatase labeled goat

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[1] Abbreviations: PBS, 0.14 M NaCl and 0.01 M sodium phosphate buffer, pH 6.8; DBM, diazobenzyloxymethyl; HMG, high mobility group chromosomal proteins; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate.

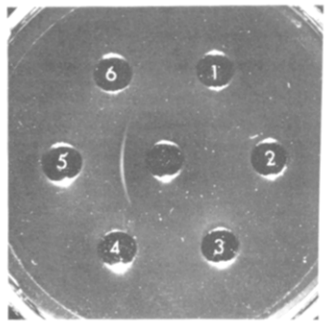

FIGURE 1: Monoclonal anti-HMG-1 are of the IgM type. Immunodiffusion in Ouchterlony plates. Center well, tissue culture fluid from cells producing anti-HMG-1; 1, anti-IgG1; 2, anti-IgG2A; 3, anti-IgG2B; 4, anti-IgG3; 5, anti-IgM; 6, anti-IgA.

anti-rabbit Ig (Kirkegaard and Perry) as described (Bustin et al., 1982). Mouse serum was tested with alkaline phosphatase labeled goat anti-mouse Ig.

Polyacrylamide Gels and DBM Transfers. Electrophoresis was performed in 18% polyacrylamide gels containing 0.1% sodium dodecyl sulfate run in Tris–glycine buffer (LeStourgeon & Rush, 1973). Transfer of proteins from the gel to DBM paper and detection of bound antibodies with ^{125}I-labeled protein A were done essentially according to Renart et al. (1979). The proteins were transferred electrophoretically in a buffer made of 0.05 M sodium phosphate, pH 6.8, and 0.05% sodium dodecyl sulfate. The transfers were made at room temperature at 1.5 A (15 V/cm) for 1 h.

Results

Monoclonal Antibodies Which React with both HMG-1 and HMG-2. The monoclonal antibody approach was used in an effort to obtain serological reagents which could readily distinguish between HMG-1 and HMG-2. Each of the six mice immunized with HMG-1 produced antibodies against this protein. Spleen cells from individual mice were fused with MS-1 myeloma cells resulting in eight wells which contained antibodies to the proteins. From these, two stable clones were isolated. Large amounts of culture media from the clones were obtained by growing the cells in roller bottles. Immunodiffusion studies indicated that the antibodies were of the IgM class (Figure 1). Ascites fluid from Balb/C mice injected with the clones was collected to obtain larger quantities of antibodies. The tissue culture supernatant and the ascites fluid were tested for anti-HMG-1 activity by ELISA and by solid phase radioimmunoassay.

A typical titration curve depicting the dependence of the amount of antibody bound on the amount of antigen added to the microtiter well is shown in Figure 2. The limit of detection using ascites fluid diluted 1:1000 and ^{125}I-labeled IgG sheep anti-mouse Ig was 250 ng of antigen. The sensitivity increased approximately 4-fold if the IgM were first reacted with IgG from rabbit anti-mouse IgM followed by ^{125}I-labeled protein A. Under these conditions the reaction is of the same magnitude as that obtained by using polyclonal antibodies elicited in rabbits and ^{125}I-labeled protein A (Figure 2). Culture fluid obtained from roller bottles in which the clones were grown gave similar results. The monoclonal antibodies did not distinguish between HMG-1 and HMG-2. The data presented in Figure 3 indicate that whereas the antibody did not react with any of the histones, it reacted strongly with both HMG-1 and HMG-2. The stronger reactivity with HMG-2 suggests that the cross-reacting determinant may be more exposed in HMG-2 or that HMG-2 binds to the plates stronger than HMG-1. The finding that the monoclonal antibodies did

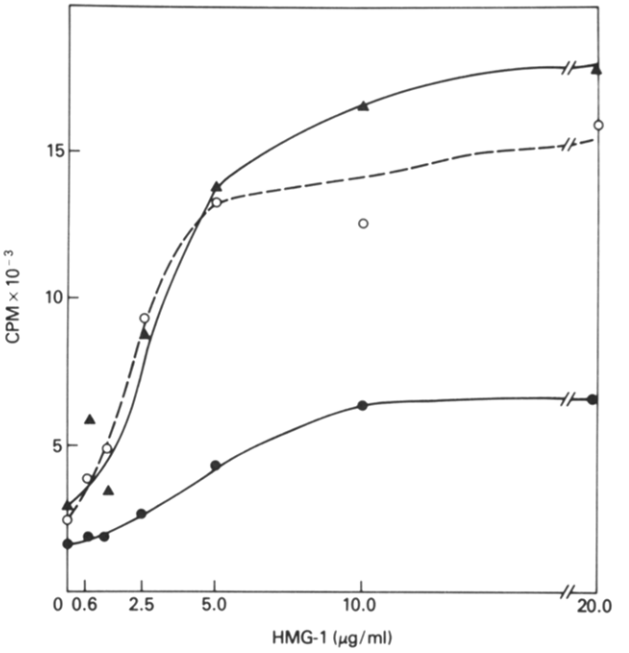

FIGURE 2: Solid phase radioimmunoassays demonstrating the dependence of antibody binding on antigen concentration. Microtiter plates were coated with 100 μL of HMG-1 at the indicated concentrations. (▲) Rabbit polyclonal antibodies diluted 1:1000 and detected with ^{125}I-labeled protein A. (●) Ascites fluid diluted 1:500 and detected with ^{125}I-labeled IgG from sheep anti-mouse Ig. (○) Ascites fluid diluted 1:500, followed by rabbit anti-mouse IgM diluted 1:500 and by ^{125}I-labeled protein A.

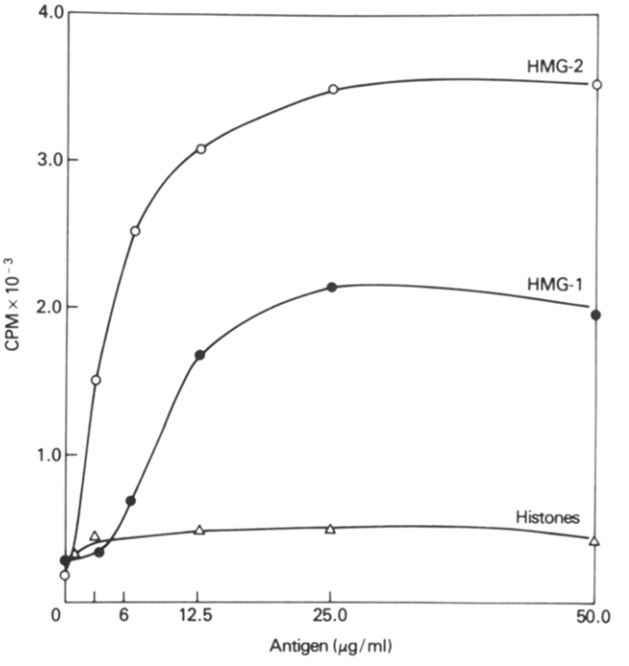

FIGURE 3: Monoclonal anti-HMG-1 does not distinguish between HMG-1 and HMG-2. Microtiter plates were coated with 100 μL of the respective antigens at the indicated concentration. Ascites fluid was diluted 1:200 for detection of HMG-1 and HMG-2 and 1:100 for testing with histones. ^{125}I-Labeled IgG from sheep anti-mouse Ig was used as second reagent.

not distinguish between HMG-1 and HMG-2 is not surprising since previous studies using polyclonal antibodies elicited by HMG-1 revealed that the two proteins are immunologically related (Romani et al., 1979, 1980; Bustin et al., 1977).

Anti-HMG-2 Sera Distinguishes between HMG-1 and HMG-2. Polyclonal antisera elicted by HMG-1 cross-react strongly with HMG-2. In contrast, the data presented in

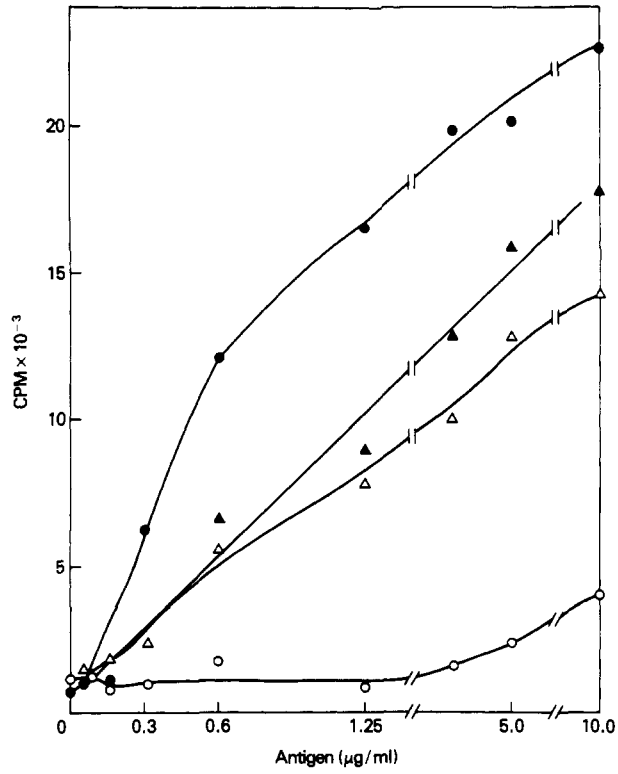

FIGURE 4: Rabbit antisera elicited by HMG-2 distinguish between HMG-2 and HMG-1. Solid phase radioimmunoassay using antisera diluted 1:500 and ^{125}I-labeled protein A. (●) Anti-HMG-2 tested with HMG-2; (○) anti-HMG-2 tested with HMG-1; (▲) anti-HMG-1 tested with HMG-1; (△) anti-HMG-1 tested with HMG-2.

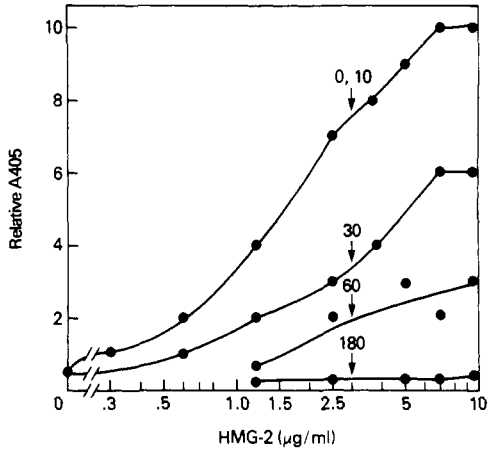

FIGURE 5: Gradual loss of antigenic activity of HMG-2 upon trypsin digestion. Aliquots from a digestion mixture were taken at various times (times of digestion in minutes indicated by the numbers over the curves.) The residual antigenic activity was tested with anti-HMG-1 diluted 1:7500 and alkaline phosphatase labeled goat anti-rabbit IgG.

Figure 4 revealed that antisera elicited by HMG-2 distinguish between HMG-1 and HMG-2.

Gradual Loss of Antigenic Determinants upon Controlled Proteolytic Digestion. The proteins were digested with trypsin and pepsin under conditions similar to those used to determine the primary sequence of the proteins to gain insights into the nature of the antigenic determinants of HMG-1 and HMG-2 (Walker et al., 1980). At various times during the digestion, aliquots were removed and assayed for residual antigenic activity by ELISA. Figure 5 depicts the gradual loss of reactivity of HMG-2 with anti-HMG-1, upon trypsin digestion. Comparison with Figure 6 indicates that, under the conditions used, pepsin digestion destroyed the antigenic determinants

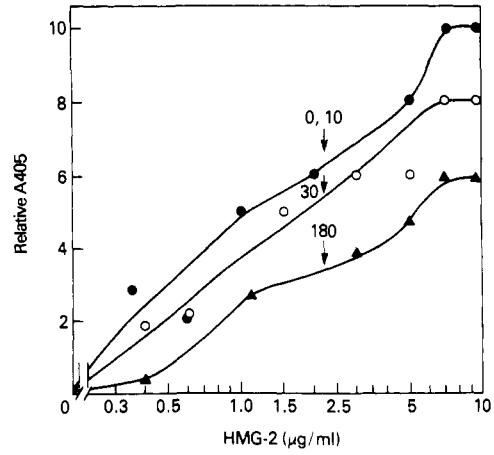

FIGURE 6: Gradual loss of antigenic activity of HMG-2 upon pepsin digestion. Residual antigenic activity tested as in Figure 5.

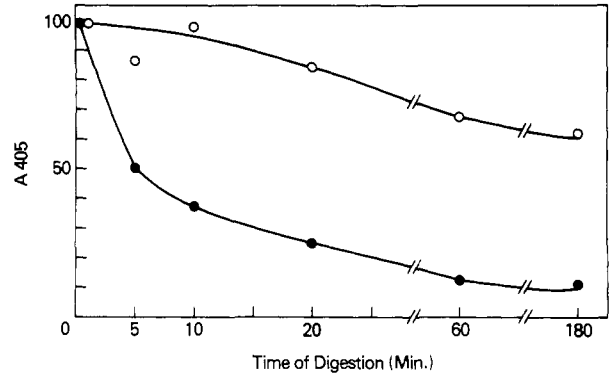

FIGURE 7: Gradual loss of antigenic activity of HMG-1 upon pepsin and trypsin digestion. Tested with anti-HMG-1 as indicated in the legend to Figure 5. (○) Pepsin; (●) trypsin.

of HMG-2 at a slower rate than trypsin digestion. A similar situation is observed when the course of loss of antigenic determinants in HMG-1 upon trypsin and pepsin digestion is followed (Figure 7). The digests of the two proteins were also tested with anti-HMG-2 and with the monoclonal antibody preparations. The rate at which HMG-2 lost the antigenic determinants reacting with anti-HMG-1 was indistinguishable from the rate at which the proteins lost the antigenic determinants reacting with anti-HMG-2. Higher concentrations of proteolytic enzymes resulted in a complete loss of antigenic determinants within 10 min. When tested with monoclonal antibodies, all reactivity was lost after 10 min of trypsin digestion or 30 min of pepsin digestion (data not shown). The gradual loss of reactivity with the polyclonal antibody could be due to either (1) residual uncleaved HMG-1 or (2) peptides which still contain intact antigenic determinants. To distinguish these two possibilities, the digest was analyzed by electrophoresis on polyacrylamide gels, the peptides were transferred from the gels to DBM paper, and the DBM paper was probed with anti-HMG-1 and anti-HMG-2.

Detection of Immunoreactive Peptides by Immunoreplica Techniques. Treatment of HMG-2 with pepsin or trypsin generates a series of polypeptides which can be resolved by electrophoresis in 18% polyacrylamide gels run in the presence of sodium dodecyl sulfate (Figure 8). Already at early stages of trypsin digestion (5 min) a series of peptides is generated. These peptides are rapidly converted to peptides of less than M_r 6000. In contrast, the pepsin digest goes through a discernible intermediate stage. A peptide of molecular weight of about 14 000 is first generated, followed by two peptides with molecular weights of approximately 13 000 and 12 000.

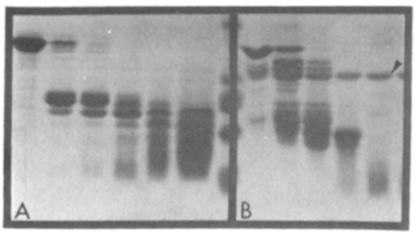

FIGURE 8: Visualization of the peptides generated from HMG-2 after various times of trypsin and pepsin digestion. Electrophoresis in 18% polyacrylamide gels run in the presenc of 0.1% $NaDodSO_4$. From left to right samples are (A) pepsin digests for 0, 2, 5, 30, 60, and 180 min and (B) trypsin digests for 0, 2, 5, 30, and 60 min.

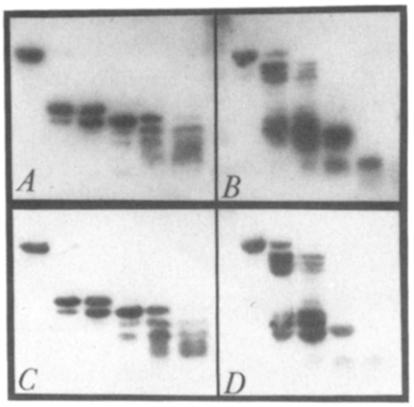

FIGURE 9: Immunoreactive peptides in HMG-2. The peptides present in a gel identical with that presented in Figure 8 were transferred to DBM paper. The paper was treated with either anti-HMG-1 or anti-HMG-2 diluted 1:200 and the bound IgG detected by ^{125}I-labeled protein A and autoradiography. The autoradiograms depict (A, C) pepsin digests and (B, D) tryptic digests. (A) and (B) were treated with anti-HMG-1 and (C) and (D) treated with anti-HMG-2.

After 1 h of digestion, a mixture of peptides all with a molecular weight smaller than 120 000 is present.

The peptides were transferred to DBM paper, and the paper was treated with either anti-HMG-1 (Figure 9A,B) or anti-HMG-2 (Figure 9C,D) followed by ^{125}I-labeled protein A. The results indicate that all the peptides, detected by Coomassie blue on the gel, bound antibodies. Anti-HMG-1 and anti-HMG-2 gave the same results. The peptides generated from HMG-1 by trypsin and pepsin (Figure 10) are similar to those observed with HMG-2. Antisera to HMG-1 reacted with all the detectable peptides (Figure 11).

Discussion

The results presented in this paper suggest that antisera elicited by chromosomal proteins HMG-1 and HMG-2 contain antibodies directed against sequential determinants present in these proteins. By definition sequential determinants are those due to an amino acid sequence in a random coil form, and antibodies to such a determinant are expected to react with peptides of identical or similar sequences. These are usually contrasted with conformational determinants whose reactivity depends on the conformation of the antigen (Sela et al., 1967; Crumpton, 1974).

The presence of sequential determinants is deduced from the finding that DBM transfers of both tryptic and peptic digests of HMG-1 and HMG-2 bind antibodies. The sequential determinants are present over different regions of the molecule since practically all the peptides visible by Coomassie blue staining of polyacrylamide gels were also detectable in the autoradiograms of the DBM paper. Furthermore, some smaller molecular weight peptides can still bind antibodies as

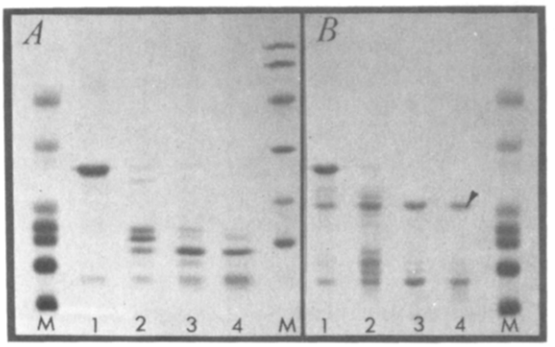

FIGURE 10: Visualization of the peptides generated from HMG-1 by controlled digestion with trypsin and pepsin. Electrophoresis as in Figure 8. Pepsin (A) and trypsin (B) digests for the following: 1, 0 min; 2, 5 min; 3, 30 min; 4, 60 min. M, molecular weight markers, at the flanks from top to bottom: ovalbumin, 43 000; chymotrypsinogen, 25 700; β-lactoglobulin, 18 400; lysozyme, 14 400; cytochrome *c*, 12 300; bovine trypsin inhibitor, 6200; insulin, 3000. Center M marker from top to bottom: phosphorylase, 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; lactalbumin, 14 400. Arrow points to soybean trypsin inhibitor.

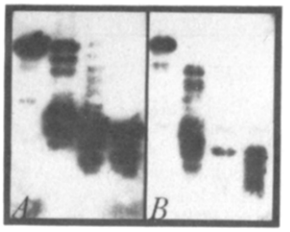

FIGURE 11: Immunoreactive peptides in HMG-1. The peptides present in a gel identical with that depicted in Figure 10 were tested for anti-HMG-1 binding as described in the legend to Figure 9.

evidenced by the finding that prolonged pepsin digestion, which generates small peptides which are lost from the polyacrylamide gels, does not fully abolish all the antibody binding activity detected by ELISA. The HMG-2 determinants which can be recognized by anti-HMG-2 are very similar to those recognized by anti-HMG-1 (see Figure 9). Anti-HMG-1 in turn recognizes similiar peptides in both HMG-1 and HMG-2. The fact that anti-HMG-2 distinguishes between HMG-1 and HMG-2 suggests that most antibodies have been elicited against peptides containing amino acid differences between these two proteins. Sequence analyses of the two proteins (Walker et al., 1980) indicated that most differences are found in the C terminal two-thirds of the molecule (after residue 70).

In view of the fact that HMG-1 elicits antibodies which cross-react with HMG-2 (Romani et al., 1979, 1980; Bustin et al., 1977), it is not surprising that the monoclonal antibodies obtained do not distinguish between these two proteins. Obviously the antibody is directed against shared antigenic determinants. During the production of hybridoma against HMG-1, we observed that it was difficult to obtain stable clones against this protein. Thus, at early stages of hybridoma production, several cell lines producing antibody to HMG-1 were detected. In many of these, the activity was lost upon subcloning. We have previously noted that antisera elicited by HMG-1 purified from calf thymus cross-react with HMG-1 purified from mouse liver (Romani et al., 1979). It is feasible that the antibody which can react with the HMG of the hybrids is toxic to the cells. The fact that HMG-1 and -2 contain several sequential antigenic determinants suggests that the polyclonal antibodies which were elicited will not be useful in detecting possible conformational changes occurring in these proteins. On the other hand this situation increases the pos-

sibility that among the various types of antibodies present in the polyclonal sera some will be able to bind to their target regardless of the exact state in which the HMG is found in the cell. Indeed, using microinjection techniques we have found that the anti-HMG-1 antibody can bind to the antigen under in vivo conditions (L. Einck and M. Bustin, unpublished results). Thus it is feasible that microinjection of functional antibodies will help elucidate the cellular role of these proteins.

Acknowledgments

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Cobalt–Bleomycin–Deoxyribonucleic Acid System. Evidence of Deoxyribonucleic Acid Bound Superoxo and μ-Peroxo Cobalt–Bleomycin Complexes†

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ABSTRACT: Co(II) interacts with bleomycin in aqueous solution, in the presence of air, to give a short-lived mononuclear superoxo Co(III) complex (I). Then, two molecules of complex I react together, with the loss of oxygen, to yield the dinuclear μ-peroxo Co(III) complex (II); the dimerization follows a second-order rate law with $k_2 = 200 \pm 50$ M^{-1} s^{-1} at 25 °C. The rate of dimerization is lowered by a factor of 2000 when DNA is present at a molar ratio of [nucleotide]/[Co] higher than 16. These results and studies of circular dichroism and electron paramagnetic resonance spectra of complexes strongly suggest the binding of the superoxo complex to DNA (I′) as well as that of the μ-peroxo complex (II′); the binding of 1 molecule of complex II for every 2.9 base pairs in DNA has been determined with an apparent equilibrium constant of 8.4×10^4 M^{-1}.

Bleomycins (BLM) are a family of glycopeptide antibiotics clinically prescribed for the treatment of selected neoplasmic diseases (Carter, 1978). This drug, which both chelates metal ions and binds to deoxyribonucleic acid (DNA), induces a degradation of DNA in a reaction that has been shown to depend, in vitro, on the presence of ferrous ion and molecular oxygen (Sausville et al., 1978). BLM can form stable complexes with other metal ions such as Cu(II), Zn(II), and Co(III) (Dabrowiak, 1980). With the exception of the Co(III) complexes, all the others have antitumor properties against the Ehrlich ascites tumor (Rao et al., 1980). On the other hand, labeled metal-bleomycin complexes are used for diagnostic tumor localization, and the Co^{III}·BLM complex, the more stable of the M·BLM complexes, exhibits excellent tumor-localizing properties in man (Rasker et al., 1975).

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