Immunochemical Detection of Chromosomal Protein HMG-17 in Chromatin Subunits†

Caroline S. M. Tahourdin, Natasha K. Neihart, Irvin Isenberg, and Michael Bustin*

ABSTRACT: Chromosomal protein HMG-17, purified from calf thymus, has been used to elicit specific antibodies in rabbits. Specific serological reaction between the antigen and the antisera is demonstrated by solid-phase radioimmunoassay and by competitive inhibition assays. The antisera did not cross-react with histones or other chromosomal HMG proteins. The antisera bound specifically to chromatin subunits isolated from HeLa cells, demonstrating that it may be used to study the in situ organization of this chromosomal protein. Chromatin purified from HeLa nuclei was digested with micrococcal nuclease, and the resulting mono- and oligonucleosomes were fractionated on a sucrose gradient. Analyses of the content of chromsomal proteins HMG-1, HMG-17, and H4 in different size nucleosomal particles, by the solid-phase radioimmunoassay, reveal that the distribution of HMG-17 was the same as that of H4, but different from that of HMG-1.

It is currently thought that components present in the nonhistone chromosomal protein fraction may be involved in aspects of gene regulation. For example, chromosomal proteins HMG-14 and HMG-17 seem to associate with transcribed genes as judged by their ability to confer DNase I sensitivity to transcribable regions of the genome (Weisbrod et al., 1980). These results imply a selective association of these HMG proteins with certain regions of the genome. Elucidation of the in situ organization of defined chromosomal components requires reagents that interact in a specific manner with the molecules complexed in their native structure. It has been demonstrated that specific antibodies can recognize antigenic sites residing in defined chromosomal proteins which are complexed in chromatin and chromosomes (Elgin et al., 1978; Bustin et al., 1978a).

The in situ organization of chromosomal protein HMG-17 is of specific interest because either this protein or its homologue is present in several kingdoms (Spiker et al., 1978), and because the protein may be specifically associated with active genes. In addition, its primary sequence has been determined (Walker et al., 1977).

We have generated and characterized anti-HMG-17 antibodies. This report presents data on the specificity of the antiserum and shows that it may be used to detect the presence of HMG-17 in chromatin subunits.

Materials and Methods

Antigens and Antisera. Chromosomal protein HMG-17 was purified from fresh frozen calf thymus by (carboxymethyl-Sephadex C25) CM-Sephadex C25 chromatography followed by preparative electrophoresis as previously described (Bustin et al., 1978b; Mardian & Isenberg, 1978). For immunization, rabbits were injected intradermally, intramuscularly, and in the foot pads with a total of 200 μg of HMG-17 in 2.5 mL of 66% complete Freund's adjuvant (Grand Island Biological); booster injections were given 2 and 3 weeks after the initial immunization. Four weeks after the initial injection, a final boost of 200 μg of protein (in 0.5 mL of 10 mM Tris-HCl, pH 7.5) was administered intravenously. Weekly bleeds were taken 1 week after the last boost and assayed by solid-phase radioimmunoassay.

Each of the four rabbits immunized produced antibodies. Antisera to chromosomal protein HMG-1 and histones H3 and H4 were prepared and purified as described earlier (Goldblatt & Bustin, 1975; Romani et al., 1979).

Solid-Phase Radioimmunoassay. Solid-phase radioimmunoassay was carried out as previously described (Romani et al., 1980), except that protein A was iodinated with Bolton and Hunter reagent (Bolton & Hunter, 1973).

Preparation of Nucleosomes from HeLa Cells. Nuclei and salt-washed nucleosomes were prepared by the method of Whitlock & Simpson (1976). In preparations in which salt washing was omitted, nuclei were taken up in 0.25 M sucrose, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) at approximately 100 A_{260} units/mL and digested with 500 units/mL micrococcal nuclease (Worthington) for 30 min at 4 °C. The reaction was terminated by addition of EDTA to 3.2 mM. The digest was then dialyzed extensively against 0.2 mM EDTA, pH 7.5, and 0.1 mM PMSF, homogenized in a tight-fitting Dounce homogenizer, and centrifuged briefly (27000*g*, 5 min). The supernatant was layered over a 12.5–45% isokinetic sucrose gradient (in 0.2 mM EDTA, pH 7.5) and centrifuged for 18–22 h at 47000 rpm in a Beckman Ti 14 rotor. Gradient fractions were pooled, concentrated, and dialyzed prior to analysis by the solid-phase radioimmunoassay. The protein and DNA composition of both individual fractions and pooled samples was analyzed by electrophoresis in polyacrylamide gels.

Polyacrylamide Gel Electrophoresis. DNA was fractionated by using neutral (Loening, 1967; Peacock & Dingman, 1967) polyacrylamide gels. Samples were either digested with proteinase K [10 μg/mL in 0.15 M NaCl, 10 mM EDTA, and 1% sodium dodecyl sulfate ($NaDodSO_4$), pH 7.0; 1 h, 37 °C] and loaded directly on the gel or digested with proteinase K and extracted with phenol/$CHCl_3$ by using a modified Marmur procedure (Britten et al., 1974).

Proteins were fractionated on 18% polyacrylamide gels by using the discontinuous $NaDodSO_4$–polyacrylamide gel system of LeStourgeon & Rusch (1973).

Transfer of Protein Bands from Polyacrylamide Gels to [(Diazobenzyl)oxy]methyl (DBM) Paper and Their Subsequent Detection by Immunological Methods. Preparation of DBM paper, gel transfer, and subsequent antibody and ^{125}I-labeled protein A incubation were effected according to Renart

† From the Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205 (C.S.M.T., N.K.N., and M.B.), and the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331 (I.I.). *Received May 23, 1980.* The work of I.I. was supported by U.S. Public Health Service Grant CA 10872.

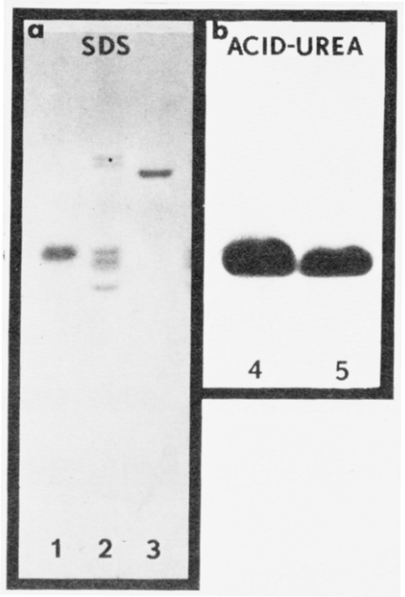

FIGURE 1: Characterization of the immunogen. (a) $NaDodSO_4$–polyacrylamide gel electrophoresis of HMG-17. A 20% polyacrylamide gel (containing 1% DATD as cross-linker) was prepared according to LeStourgeon & Rusch (1973). (Lane 1) HMG-17; (lane 2) HeLa mononucleosome; (lane 3) chicken erythrocyte specific H5. (b) Acid–urea gel electrophoresis of HMG-17. A 15% polyacrylamide gel was prepared according to Panyim & Chalkley (1969). (Lanes 4 and 5) HMG-17.

et al. (1979). Polyacrylamide gels were prepared as before, but the reversible cross-linker *N,N'*-diallyltartardiamide (DATD) was used instead of bis(acrylamide) (Anker, 1970). DATD was used at a ratio of 1 part DATD to 20 parts acrylamide. Gels were "relaxed" by incubation in 2% periodic acid (neutralized to pH 7.0) for 30 min, and then they were washed for 15 min each in 500 mM sodium borate buffer, pH 8.0, followed by 15 min in 50 mM sodium borate buffer, pH 8.0.

Results

The Antigen. Chromosomal protein HMG-17 was electrophoresed on polyacrylamide gels run in either 6 M urea or 0.1% sodium dodecyl sulfate. From Figure 1, it can be seen that the protein migrated as a homogeneous molecular species. Its electrophoretic mobility and amino acid composition (not shown) were similar to that reported by Johns and co-workers (Rabbani et al., 1978).

Detection of Specific Binding of Anti-HMG-17 Sera to HMG-17 Protein. The interaction between chromosomal protein HMG-17 and its antiserum was detected by the solid-phase radioimmunoassay recently described (Romani et al., 1980). Briefly, this assay involves adsorption of the antigen to microtiter plates, addition of antiserum, and detection of the amount of specific IgG bound by ^{125}I-labeled protein A. The dependence of antibody binding on antigen concentration is presented in Figure 2. At 1:100 sera dilution, antigen saturation is reached at about 1 μg/mL HMG-17. However, this test easily detects the protein present in 50 μL at a concentration as low as 0.03 μg/mL, which is equal to 1.5 ng of HMG-17 per microtiter well. Studies on the dependence of antibody binding on antiserum concentration (not shown) indicated that the binding is specific over sera dilutions ranging from 1:50 to 1:3000.

During these studies, we noted that, unlike other chromosomal proteins tested (M. Bustin and N. K. Neihart, unpublished experiments), the reaction between HMG-17 and anti-HMG-17 is very sensitive to the condition under which

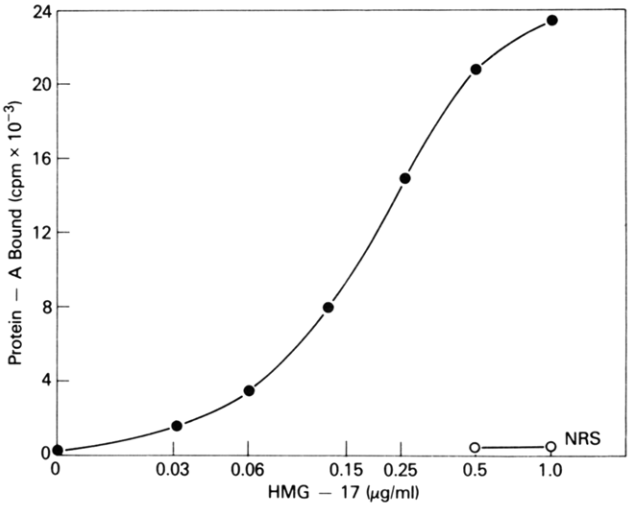

FIGURE 2: Solid-phase radioimmunoassay for HMG-17. Dependence of antibody binding on antigen concentration. A 100-μL antigen solution at each concentration was added to individual microtiter plate wells. Antiserum was added at 1:100 dilution. (●) Anti-HMG-17; (○) normal rabbit serum at the same dilution.

Table I: Immunological Specificity of HMG-17[a]

antigen in well	concn (μg/mL)	antisera used	dilution	counts bound (%)
HMG-17	1.0	HMG-17	1:100	100
HMG-1	1.0	HMG-17	1:100	2
histones	1.0	HMG-17	1:100	3
F-actin	1.0	HMG-17	1:100	1
HMG-17	1.0	preimmune	1:50	0
HMG-17	1.0	HMG-1	1:50	1
HMG-17	1.0	histone H1	1:50	0
HMG-17	1.0	histone H2A	1:100	0
HMG-17	1.0	histone H2B	1:100	0
HMG-17	1.0	histone H3	1:100	0
HMG-17	1.0	histone H4	1:100	0
HMG-17	1.0	histone H5	1:100	0

[a] Tested by the solid-phase radioimmunoassay as described under Materials and Methods.

the protein is added to the microtiter wells. Thus, when the pH of the solution in which the protein is added is below 7.2, over 75% of the antibody binding capacity is lost. This is not due to irreversible conformational change in the protein because preexposure of the protein to pH as low as 2.5 followed by readjustment to pH values higher than 7.4 results in 100% binding. Similarly, the antibody–antigen reaction is highly dependent on the NaCl concentration of the protein solution applied to the microtiter plates. The binding obtained with solutions of 0.35 M NaCl or higher was only 25% of that obtained when the protein was applied to the plates in 0.15 M NaCl.

The data compiled in Table I further document the specificity of the antiserum. Compared to immune serum, preimmune serum, antiserum to nonhistone chromosomal protein HMG-1, or antiserum to histone H1 displayed only 1% protein A binding activity. Similarly, negligible protein A binding was observed when the microtiter wells were coated with other chromosomal proteins such as HMG-1, each of the histones, or actin and tested with anti-HMG-17.

Presence of HMG-17 in Chromatin Subunits. Chromatin subunits were isolated from HeLa nuclei that had been digested with micrococcal nuclease (see Materials and Methods). These nucleosomes were used as antigens and tested for their ability to bind anti-HMG-17 sera. The data presented in

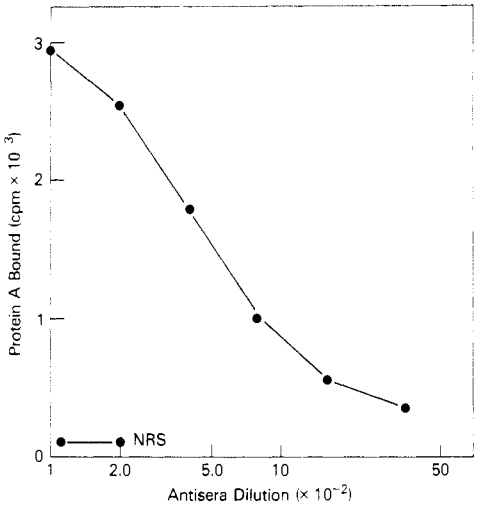

FIGURE 3: Binding of anti-HMG-17 sera by chromatin particles purified from HeLa cells. Solid-phase radioimmunoassay for HMG-17 in mononucleosomes prepared from HeLa cells; dependence of antibody binding on antisera concentration. Serial dilutions of anti-HMG-17 antiserum were added to microtiter plate wells pretreated with 500 ng of mononucleosomes. (●) Anti-HMG-17; (● NRS) normal rabbit serum.

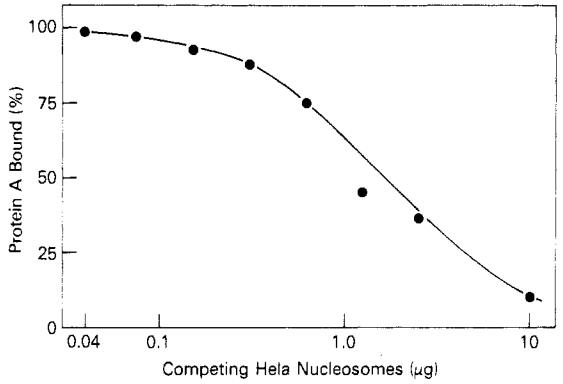

FIGURE 4: Inhibition of anti-HMG-17 binding to immobilized HeLa nucleosomes by preincubation with free nucleosomes and detection by solid-phase radioimmunoassay. Antiserum at 1:400 dilution was incubated (18 h at 4 °C) with an equal volume of 1% bovine serum albumin in phosphate-buffered saline containing increasing amounts of antigen. After centrifugation, the supernatant was added to microtiter plate wells pretreated with 50 ng of nucleosomes. Protein A bound expressed as percent of control (no inhibitor).

Figure 3 indicate that the antiserum bound to the chromatin subunits. Nucleosomes which were washed with solutions containing 0.4 M NaCl lost 80% of their ability to bind this antiserum. The reaction between the nucleosomes and the antiserum could be inhibited by preincubation of the antiserum with the nucleosomes (Figure 4). Interestingly, the nucleosomes served as a better competitor than free HMG-17. Under the conditions of the assay described in Figure 4, 50% inhibition was obtained with 1.8 μg of nucleosomes. An equal amount of HMG-17 reduced the binding by only 10–20%. The fact that the nucleosomes-bound HMG-17 is a more efficient inhibitor than free HMG-17 could be explained as follows: in the solid-phase radioimmunoassay, inhibition of binding will be detected only if both binding sites of the antibody are prevented from interaction with the antigen that coats the microtiter wells (Romani et al., 1980). Competition experiments with chromatin particles may present a situation where binding of the relatively bulky chromatin particle to one antibody binding site prohibits interaction of the second antibody binding site to the immobilized antigen, due to steric hindrance. Because HMG-17 is a small protein, such steric hindrance may

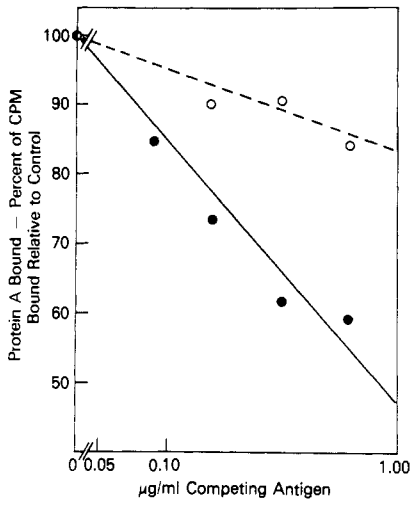

FIGURE 5: Inhibition of anti-HMG-17 binding to immobilized HMG-17 by preincubation with HeLa nucleosomes or HMG-17 and detection by solid-phase radioimmunoassay. Antiserum at 1:100 dilution was incubated with increasing amounts of antigen; after centrifugation, the supernatant was added to microtiter plates pretreated with 50 ng of HMG-17 (see Figure 4 for details). (○, - - -) Nucleosome as competing antigen; (●, —) HMG-17 as competing antigen. Data expressed as percent protein A bound relative to control.

not occur when it, rather than a nucleosome, is used as an inhibitor. Analternative explanation is that the antigen–antibody complex is only partially removed by centrifugation, and the remainder binds to immobilized nucleosomes (which may already have an antibody bound to the other antibody binding sites). That HMG-17 is present in HeLa nucleosomes is also substantiated by the converse experiment (Figure 5) where HMG-17 served as the immobilized antigen. In this case, where the effects of steric hindrance are less pronounced, the inhibition is dependent on the absolute amount of HMG-17. Obviously, for a given weight, free HMG-17 is a significantly better inhibitor than nucleosomes.

The presence of authentic HMG-17 in the chromatin particle was demonstrated as described below. The proteins present in HeLa nucleosomes were fractionated by electrophoresis in a 20% polyacrylamide gel cross-linked with the reversible cross-linker DATD and run in the presence of 0.1% sodium dodecyl sulfate. Proteins were transferred from the gel and covalently bound to [(diazobenzyl)oxy]methyl paper (Renart et al., 1979). The paper was reacted with either antisera to HMG-17 or anti-HMG-1. The location of the bound antibody was visualized by reaction with ^{125}I-labeled protein A followed by autoradiography. The data are presented in Figure 6, lower panel. The Coomassie blue stain of the transferred gel indicates the location of the chromosomal proteins in the HeLa nucleosomes and of protein markers. The autoradiogram of the paper after treatment with anti-HMG-17 indicates that only the HMG-17 marker and the HMG-17 in the nucleosomal protein reacted with the antibody. Also shown in the lower panel is the binding of anti-HMG-1 to the same paper; it may be seen that only the marker protein binds antiserum. This demonstrates the difference in the binding of HMG-1 and HMG-17 to nucleosomes, for whereas it is possible to detect HMG-17 in nucleosomes prepared in the presence of 0.15 M NaCl, these particles lack HMG-1. We have previoulsy noted that in this electrophoretic system HMG-17 migrates in the same position as histone H3 (Bustin et al., 1978b). The absence of any cross-reaction between H3 and HMG-17 is demonstrated in the upper panel.

The distribution of the HMG-17 protein among chromatin stretches of various sizes was assessed by measuring the relative

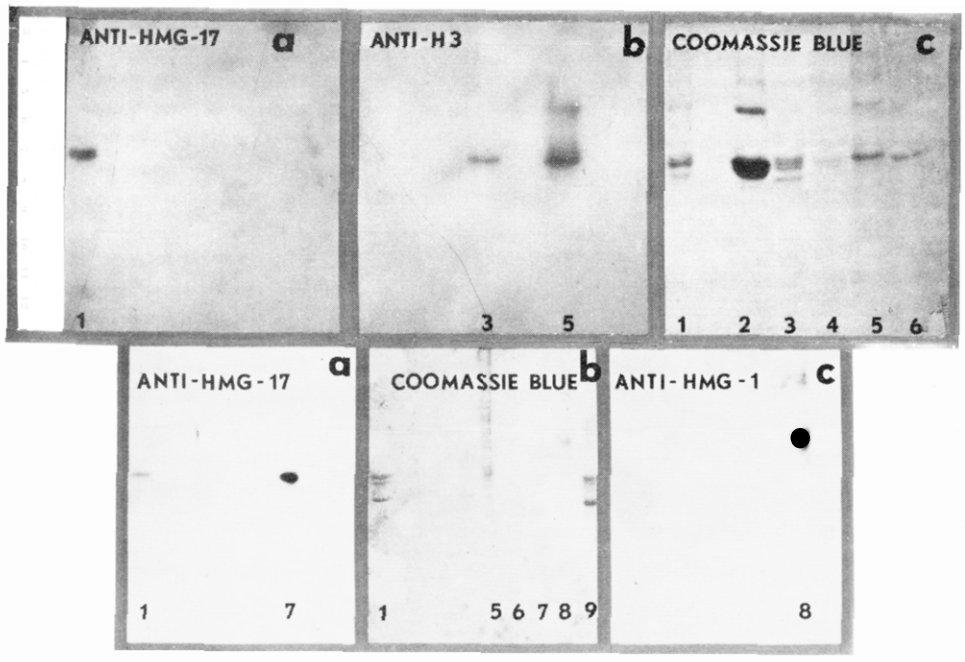

FIGURE 6: Immunological detection of proteins fractionated by $NaDodSO_4$–polyacrylamide gel electrophoresis on 20% acrylamide/DATD gels (see Materials and Methods). Gels were transferred to DBM paper and incubated in turn with each antiserum at 1:100 dilution. Removal of the first antiserum was effected by incubating the DBM paper in 10 M urea, 50 mM sodium phosphate, pH 7.0, and 0.1 M mercaptoethanol for 1 h at 60 °C. *Top panel: HMG-17 and H3 have very similar* electrophoretic mobilities but may be distinguished immunologically. Autoradiograms of DBM paper incubated in anti-HMG-17 (a) and anti-H3 (b). (c) Coomassie blue stain of the original gel after transfer: (lane 1) fractionated calf thymus histones H2A and H4, and nonhistone chromosomal protein HMG-17; (lanes 2 and 4) partially fractionated chicken erythrocyte histones; (lane 3) proteins from H1/H5 stripped chicken erythrocyte nucleosome; (lanes 5 and 6) fractionated calf thymus histones H3 (lane 5) and H2B (lane 6). Lower panel: HeLa nucleosomes contain HMG-17. Autoradiograms of DBM paper incubated in anti-HMG-17 (a) and anti-HMG-1 (c). (b) Coomassie blue stain of gel after transfer to DBM paper (high background due to 3MM paper adhering to the gel): (lane 1) proteins from HeLa nucleosomes; proteins in lanes 2, 3, and 4 were unrelated samples; (lane 5) proteins released into supernatant during predigestion of HeLa nuclei with nuclease [see Whitlock & Simpson (1976)]; (lane 6) chicken erythrocyte H1 + H5; (lane 7) calf thymus HMG-17; (lane 8) calf thymus HMG-1; (lane 9) H3 + H4 from chicken erythrocytes.

content of HMG-17 in different sucrose gradient fractions from a micrococcal nuclease digest of HeLa nuclei (prepared in the absence of salt). The distribution of HMG-17 was compared to that of chromosomal protein HMG-1 and of histone H4. The micrococcal nuclease digest was fractionated in a Ti 14 zonal rotor on a 12.5–45% isokinetic sucrose gradient. The length of DNA present in each fraction was determined by electrophoresis in neutral 5% polyacrylamide gels. Appropriate fractions were pooled, concentrated, and probed for HMG-17, HMG-1, and H4 content by the solid-phase radioimmunoassay (Romani et al., 1980). The data are present in Figure 7. The sedimentation pattern obtained is typical of a nuclease digest. The radioimmunoassay revealed that a large proportion of HMG-1 remained at the top of the gradient, unassociated with chromatin subunits. Histone H4 and HMG-17, however, were not released and remained associated with nucleosome monomer and multimers. The monomer region bound equivalent amounts of anti-HMG-1 and anti-HMG-17 and significantly more anti-H4. Analysis of dinucleosome and faster sedimenting material (tube 35 and up) showed no change in the relative amounts of the three antigens tested. It is noteworthy that some nucleosome monomers still contained HMG-1. These results are in agreement with previous findings that micrococcal nuclease digestion brings about a preferential release of protein HMG-1 (which in most cases is bound to the linker region), that HMG-17 remains associated with nucleosomes, and that nucleosome monomers are heterogeneous in their content of HMG-1 (Goodwin et al., 1977).

In an attempt to visualize HMG-17 by indirect immunofluorescence, HeLa, human KD, and bovine EBTR cells were fixed by a variety of methods (ethanol/methanol and/or

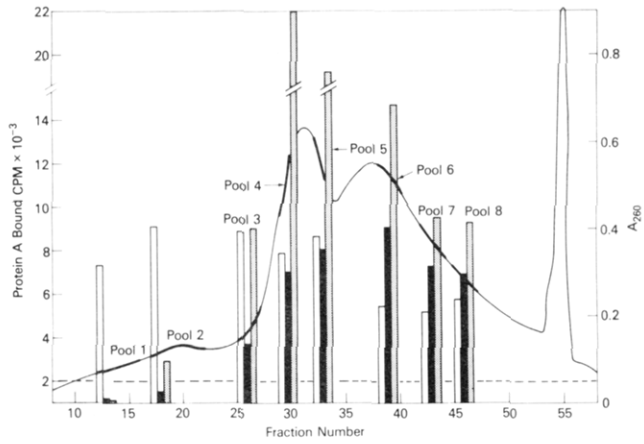

FIGURE 7: Relative distribution of nonhistone chromosomal proteins HMG-1 and HMG-17 and histone H4 assayed across a sucrose gradient used to fractionate a micrococcal nuclease digest of HeLa nuclei. HeLa nuclei were digested for 30 min at 4 °C, fractionated on a 12.5–45% sucrose gradient (see Materials and Methods), and pooled as indicated. Samples (100 μL) from each pool were added to microtiter plate wells and probed with anti-HMG-1 (open bar), anti-HMG-17 (black bar), or anti-H4 (dotted bar) at an antiserum dilution of 1:100. Dotted line shows nonspecific values obtained by using normal rabbit serum.

acetone, both with and without formaldehyde cross-linking) and probed with anti-HMG-17 and FITC-labeled protein A. None of the cells displayed significant fluorescence. Since the antibodies can recognize HMG-17 complexed in its nucleosomal conformation, we excluded the possibility that the absence of fluorescence was attributable to conformational changes between the HMG-17 molecules free in solution and

in their nucleosome-bound state. However, since the HMG-17–anti-HMG-17 reaction may be sensitive to alterations in pH and ionic strength, this may explain the lack of any specific fluorescence in situ. Other possible explanations for the lack of fluorescence are the following: (a) the concentration of HMG-17 is too low for detection by this method (HMG-1, which is detected by this method, is a significantly more abundant protein in these tissues); (b) the HMG-17 is extracted in spite of fixation; (c) HMG-17 antigenic determinants are buried and not accessible to the antiserum.

Discussion

The results presented in this manuscript indicate that chromosomal protein HMG-17 elicits specific antibodies and that these antibodies can recognize the antigenic determinants of an HMG-17 molecule which is complexed in the nucleosome.

The specificity of anti-HMG-17 corresponds well with that observed by us for other chromosomal proteins. Thus, antisera elicited by histones and protein HMG-1 do not react with HMG-17. Polyacrylamide gel electrophoresis of chromosomal proteins from various sources revealed that most tissues examined contain a polypeptide chain with a mobility identical or closely similar to chromosomal protein HMG-17 purified from calf thymus (Rabbani et al., 1978, 1980; Walker & Johns, 1980). The present study reveals that the HMG-17 present in HeLa cells is also similar antigenically to its homologue from calf thymus. Thus, HMG-17 displays little tissue specificity, at least within the animal kingdom. This lack of specificity suggests that certain structural features in this protein, conserved during evolution, are vital to certain cellular processes.

The finding that antisera elicited by HMG-17 purified from calf thymus cross-react with HMG-17 from HeLa cells suggests that one type of antiserum can be used as a reagent to study the in situ organization of this protein in a variety of experimental systems. The advantages of using a heterologous system in serological studies of chromosomal components have already been pointed out (Bustin, 1977).

Analysis of the distribution of HMG-17 in sucrose gradient fractions of a nuclease digest suggests that this protein is not released from the chromatin subunits and it always remains associated with intact particles. The sedimentation profile presented in Figure 7 is obtained from a preparation which was digested to over 30% acid solubility. The digest was dialyzed extensively against 0.25 mM EDTA, pH 7.2, prior to fractionation on a sucrose gradient. Since this sample was extensively degraded, we expected a population of subnucleosomal particles generated by nuclease breaks within the nucleosome and, therefore, expected that the HMG-17 associated with these particles would be released and appear with the slowly sedimenting material at the top of the sucrose gradient. This was not the case; hence, we suspect the HMG-17 released during digestion reassociated with the undigested fraction. Alternately, chromatin regions containing HMG-17 may be particularly resistant to micrococcal nuclease digestion. By the same reasoning, it appears that at least part of the HMG-1 stays at the top of the sucrose gradient and does not reassociate with undigested regions. Considerable quantities of HMG-1 are released early in the course of a micrococcal nuclease digestion, lending support to the claim that this protein is present in the internucleosomal linker region (Levy et al., 1977). HMG-1 has also been detected associated with nucleosomal core particles; evidence from several laboratories suggests this is not due to reassociation of free protein with the core (Mathew et al., 1979; Jackson et al., 1979; Levy et al., 1979). Jackson et al. (1979) and Levy et al. (1979) both describe procedures for fractionating HMG-1- (+HMG-2-) containing nucleosomes from the bulk of the population. Mathew et al. (1979) propose a homogeneous distribution for HMG-1 but postulate that there are two or more subpopulations of these proteins; our data would be consistent with this notion. Analyses of fractions obtained from a brief micrococcal nuclease digest gave similar results: HMG-1 was present at the top of the gradient while HMG-17 was associated with the chromatin fragments. It is noteworthy that analysis of the protein content of different fractions from the sucrose gradient by the solid-phase radioimmunoassay is significantly more convenient and requires substantially less material than the conventional approach, which relies on electrophoresis in a polyacrylamide gel.

It is now possible to introduce functionally active IgG molecules into cells. Thus, microinjection of antibodies to histones into oocytes of *Pleurodeles waltlii* resulted in loss of transcription loops (Scheer et al., 1979). Microinjection of antiactin into the germinal vesicle of *Xenopus laevis* oocytes specifically inhibited chromosome condensation (Rungger et al., 1979). Recent results suggest that chromosomal protein HMG-17 is associated with transcribably regions of the genome (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980; Levy & Dixon, 1978; Levy et al., 1979; Bakayev et al., 1979). The availability of antibodies specific to this protein opens the possibility of examining its role in a functional cell and may facilitate isolation of chromosomal regions containing HMG-17.

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High-Potential Iron–Sulfur Proteins and Their Possible Site of Electron Transfer†

George Aprahamian and Benjamin A. Feinberg*

ABSTRACT: The electron-transfer mechanism of the Fe_4S_4* high-potential iron–sulfur proteins (HiPIP's) was explored via a stopped-flow spectrophotometric kinetic study of the reduction of *Chromatium vinosum* and *Rhodopseudomonas gelatinosa* HiPIP's by both native and trinitrophenyllysine-13 horse cytochrome *c*. The influence of electrostatic effects was also effectively partitioned from the redox process per se. The corrected rates were 12.3×10^4 and 3.8×10^4 M^{-1} s^{-1} for native with *C. vinosum* and *R. gelatinosa* HiPIP, respectively, and 17.5×10^4 and 5.46×10^4 M^{-1} s^{-1} for TNP-cytochrome *c* with the two HiPIP's, respectively. The faster rates of TNP-cytochrome *c* with the HiPIP's are unexpected in terms of possible steric interaction since lysine-13 is at the top of the heme crevice. In understanding the somewhat faster rates of the TNP-cytochrome *c* over native cytochrome *c* it is possible that (1) TNP-cytochrome *c* reacts more quickly since modification of the lysine-13 residue destabilizes somewhat the heme crevice or (2) in light of the hydrophobic nature of the trinitrophenyl group and the X-ray crystallographic structure of HiPIP, the TNP group facilitates electron transfer by interacting with a hydrophobic region on the HiPIP molecular surface. The region about the S*4 sulfur atom is the most exposed and accessible hydrophobic region on the HiPIP surface, in addition to being the point of closest approach of the S*4 to the external environment.

Much effort has been expended in the past decade in the determination of the oxidation–reduction mechanisms of the important classes of redox proteins, including the cytochromes *c*, flavoproteins, copper, and iron–sulfur proteins. Besides the study of the biological redox couples, redox protein mechanism has also been approached via the nonphysiological study of small molecule–protein reactions and to a much lesser extent biologically nonspecific protein–protein reactions. Nonspecific protein–protein redox reactions, however, are of particular interest since they ensure with much greater certainty that an outer-sphere electron-transfer mechanism will prevail (most probable for biological protein–protein reactions) and, for this reason, also mimic more closely the biological protein–protein reactions than the small molecule–protein reactions.

In this study the electron-transfer mechanism of the high-potential iron–sulfur proteins (HiPIP's)[1] is explored via a stopped-flow spectrophotometric kinetic study of the reduction of *Chromatium vinosum* and *Rhodopseudomonas gelatinosa* HiPIP's by both native and trinitrophenyllysine-13 horse cytochrome *c*. The TNP group is both particularly bulky and hydrophobic, and modification of the lysine-13 residue provides an especially interesting derivative of cytochrome *c* since it might be expected to partially block the heme crevice region where electron transfer is generally demonstrated to take place. Additionally, when lysine-13 is TNP modified, it does not transfer electrons to cytochrome *c* oxidase since the specific binding between the two molecules is disrupted (Smith et al., 1977; Ferguson-Miller et al., 1978). Lastly, in this work the electrostatic contributions to the redox rates have been effectively partitioned from the electron-transfer process per se to obtain electrostatically corrected rate constants. The need and usefullness of such corrections have been demonstrated (Feinberg & Johnson, 1980; Ilan et al., 1979); moreover, such corrections provide a clearer view of the HiPIP electron-transfer mechanism.

† From the Department of Chemistry and The Laboratory for Molecular Biomedical Research (Contribution No. 117), The University of Wisconsin—Milwaukee, Milwaukee, Wisconsin 53201. *Received June 30, 1980.* This work was performed with the support of the Science and Education Administration of the U.S. Department of Agriculture under Grant No. 5901-0410-8-0156-0 from the Competitive Research Grants Office, The Research Corporation, and The University of Wisconsin—Milwaukee Graduate School Research Committee.

[1] Abbreviations used: HiPIP, high-potential iron–sulfur protein; TNP, trinitrophenyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; phen, phenanthroline.

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