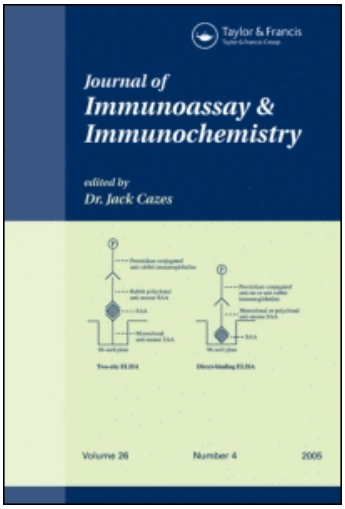

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Solid-Phase Enzyme Immunoassay for the Detection of HMG Nonhistone Proteins in Their Native Structure

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SOLID-PHASE ENZYME IMMUNOASSAY FOR THE DETECTION OF HMG NONHISTONE PROTEINS IN THEIR NATIVE STRUCTURE

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ABSTRACT

The use of purified specific antibodies against HMG1 and HMG17 (high mobility group nonhistone chromosomal proteins), together with a very sensitive solid phase enzyme immunoassay, allows the detection of nanograms of these proteins free in solution or in chromatin native structure, and the measurement of their accesibility. The sensitivity of the assay is comparable to that of the radioimmunoassay, with the advantage of avoiding the handling of radioactive materials.

(KEY WORDS: Bioassays, immunochemical methods, ELISA, immunoglobulin purification, protein conformation, chromatin.)

INTRODUCTION

The structure of chromatin and its function depends on the interactions between the different components. However, the functionality of chromosomal proteins is not easy to assay; a good approach to the structure and function of these proteins is the use of specific antibodies and immunochemical assays. Several techniques have been used up to now: immunosedimentation (1), complement fixation (2) and radioimmunoassay (3) are among the most suitable techniques for defining conformational relatedness

between histones or nonhistones. A new solid phase enzyme immunoassay is presented in this report, with a sensitivity comparable to that of a solid phase radioimmunoassay used in the detection of chromosomal components (4). An enzyme-linked immunosorbent assay (ELISA) has been previously applied to the study of histone components in nucleosome structure (5), but its sensitivity was 10 fold less than that of RIA. In the ELISA method described here, the use of horseradish peroxidase (HRP), together with the substrates DMAB-MBTH as chromogenic system, introduced by Ngo and Lenhoff (6), allows the detection of very small quantities of protein, as low as 10 ng/ml.

The aim of this paper is to describe an improved ELISA, first developed by Nieto et al. (7), for the detection of small quantities of HMG nonhistone proteins (HMG1 and HMG17), in solution or in native chromatin.

MATERIALS AND METHODS

Immunization Procedure

HMG nonhistone proteins were obtained from calf thymus by means of a modification of the method by Sanders (8). HMG1 and HMG17 were extracted with 5% percloric acid and fractioned precipitation with acetone. Samples were purified by ion-exchange chromatography, and tested by polyacrylamide gel electrophoresis (PAGE-SDS).

Female New Zealand white rabbits were immunized with HMG proteins dissolved in Tris-HCl buffer 10 mmol/l, pH 8.8 and emulsified in complete Freund's adjuvant (1:1 v/v). Each animal received subcutaneous and intramuscular injections on days 1, 8 and 15; the dose used was 0.4 mg of pure protein in a total volume of emulsion of 1.4 ml. Booster injections, separated by periods of at least one month, were given. The animals were bled by direct cardiac puncture; usually we obtained 10-15 ml of serum per bleeding.

Purification of Specific Antibodies by Affinity Chromatography

Specific anti-HMG1 and anti-HMG17 antibodies were obtained as follows: an affinity chromatography gel was prepared by coupling 7 ml of CNBr-activated Sepharose 4B (Pharmacia) to HMG proteins (HMG1 or HMG17), dissolved in bicarbonate buffer 100 mmol/l, pH 8.3, with NaCl 500 mmol/l, at a final concentration of 1.2 g protein/l.

The washed suspension of the protein-coupled gel was incubated with a pool of antisera, to which was added phenyl methyl sulfonyl fluoride (PMSF), 0.5 mmol/l. The sera were cycled twice through a chromatography column. Two eluting methods for the recovery of the bound antibodies were used: an acid treatment with glycine-HCl 200 mmol/l, pH 2.8, with 0.05% Tween-20, and a chaotropic treatment with KSCN 2 mol/l, containing 0.05% Tween-20, in PBS (Phosphate-buffered saline, phosphate buffer 100 mmol/l, with NaCl 140 mmol/l, pH 7.2). The fractions collected were brought to neutral pH, dialyzed against PBS, and finally stored at −20°C.

Determination of the Antibody Class

In order to determine the immunoglobulin class of the affinity-purified anti-HMG antibodies, an immunodiffusion in Ouchterlony plates was performed by using a standard anti-rabbit IgG goat antibody (Miles). The plates, containing 1% agarose in Tris-acetic buffer 80 mmol/l, pH 8.6, were incubated for 48 h at room temperature, and stained with Coomasie blue.

The immunoglobulin class present in the solutions of anti-HMG antibodies was also determined by means of PAGE-SDS. This method allows estimation of the quality of the preparative method and the purity of the samples. The antibody solutions were analyzed by gradient electrophoresis (3-20% acrylamide), under nonreducing conditions, so that the immunoglobulin molecules were not dissociated in their subunities. The low density of the gel in its upper section makes it possible the entry of any high molecular weight IgM antibodies.

Enzyme Immunoassay

The experimental procedure for the enzyme-linked immunosorbent assay (ELISA) has been described elsewhere (9, 10). Briefly, polystyrene microtiter plates were coated by overnight incubation with HMG proteins in carbonate buffer 100 mmol/l, pH 9.6, at room temperature. The protein concentration of the HMG solutions was determined spectrophotometrically, using the following molar extinction coefficients: HMG1, ϵ_{280} = 20900 $l \times mol^{-1} \times cm^{-1}$; HMG17, ϵ_{280} = 25400 $l \times mol^{-1} \times cm^{-1}$ (11). Plates were then rinsed three times with PBS, with 0.05% Tween-20. The remaining active groups of the polystyrene were blocked for 1 h at room temperature with 200 µl per well of PBS, with 1% BSA (bovine serum albumin) and 0.05% Tween-20. The anti-HMG antibody solutions were diluted in the same blocking buffer and then incubated with the antigen for 2 h at 37°C (200 µl/well). After rinsing three times, an anti-rabbit IgG-HRP conjugate (Bio-Rad) diluted 1/3000 was added (200 µl/well) and incubated for 2 h at 37°C. The excess was rinsed again. The bound enzyme conjugate was detected by adding substrate solution: 3-dimethylaminobenzoic acid (DMAB) 40 mmol/l, 3-methyl-2-benzothiazolinone hydrazone (MBTH) 0.8 mmol/l, and H_2O_2 3 mmol/l in phosphate buffer 100 mmol/l, pH 7. The absorbance at 600 nm was read in a Titertek Multiskan microplate reader.

The titration of the antibodies purified by affinity chromatography was carried out by ELISA. The titer was defined as the dilution of antibody for 50% of the maximum absorbance. The measurement of cross-reactivity against the heterologous HMG proteins, and an inhibition assay with native chromatin, were also performed by ELISA.

Measurement of the Cross-reactivity

Because of the close structural relationship between HMG1 and HMG2 proteins, polyclonal antibodies raised against HMG1 will cross react with HMG2. But for most experimental purposes, it is desirable to avoid any cross-reaction between HMG1 and HMG17.

Therefore, each antibody was tested by ELISA against the homologous and the heterologous antigen. The absence of cross-reactivity was also checked by blotting the antigens to nitrocellulose (NC) membranes, and performing the following immunodetection procedure: PAGE-SDS of the HMG proteins was carried out. The antigens were then blotted to NC membranes by mass flow of solvent, using glycine 150 mmol/l, with 20% methanol in Tris-HCl buffer 25 mmol/l, pH 8.3. The transfer proceeded for 24-48 h at room temperature. The procedure for immunodetection of the blotted HMG proteins was as follows: NC membranes were blocked for 1 h using PBS with 3% BSA, then incubated for 1 h with the anti-HMG antibodies diluted in 1% BSA, PBS. After washing with PBS containing 0.05% Tween-20, membranes were incubated for 1 h with anti-IgG-HRP. The bands where the first antibody was bound were detected by addition of colour development reagent, containing 4-chloro-1-naphthol (Bio-Rad) 0.5 g/l in PBS, with 0.01% H_2O_2.

Detection of HMG Proteins in Native Chromatin

Rat liver chromatin was digested by the method of Tata and Baker (12) and dialyzed against triethanolamine buffer 10 mmol/l, pH 7.5, with NaCl 100 mmol/l, dithiothreitol 2 mmol/l, $CaCl_2$ 0.25 mmol/l and 8% glycerol. The final protein concentration of the chromatin digest was determined spectrophotometrically. Dilutions were prepared at 0.5, 1 and 5 mg/l. Chromatin samples were preincubated 1:1 (v/v) with the diluted antibody solutions for 2 h at room temperature.

On the other hand, an ELISA with HMG1 or HMG17 as microplate coated antigens was performed, using several antigen concentrations (from 0.025 to 1 mg/l). All the steps of the assay were carried out as described above, using anti-HMG specific antisera previously incubated with chromatin as first antibody.

RESULTS

Affinity Purified Antibodies

With the preparative method for anti-HMG antibodies described above, we achieved a yield of 0.08 g of specific anti-HMG

antibody/l of serum. The combination of both types of treatment (acid and chaotropic) increases the yield of the elution procedure in recovering antibodies, probably by eluting the most tightly bound fraction.

The solutions of antibody obtained after dialysis showed protein concentrations, determined spectrophotometrically, between 0.1 and 0.2 g/l, assuming that 1 g/l of antibody corresponds to an A_{280} of 1.5 (13). Figure 1 shows the analysis of these solutions by means of PAGE-SDS in 3-20% acrylamide gradient. It can be seen that the major antibody class present in the samples is IgG. The figure also shows the quality of the preparative method, since no impurities are observed. The immunodiffusion analysis of antibody samples in agarose gels (Fig. 1) confirms that the antibodies belong to the IgG class of immunoglobulins.

Enzyme Immunoassay of HMG proteins

In order to achieve the best reproducibility in ELISA, it is important to find out the antigen concentration at which it adsorbs as a monolayer on the polystyrene surface of the plate. When protein concentration is high, protein-protein interactions rather than protein-plastic interactions predominate, and reproducibility decreases. If the concentration is low, a loss of sensitivity is observed. The optimal point of antigen concentration was determined by assaying a large range of HMG concentrations (between 0.01 and 10 mg/l) against a dilution of antibody of known good response. Plotting the colour obtained in the assay versus the concentration of antigen added to the well, the point where the plateau is achieved is the optimal amount of antigen per well in the assay, or saturation point.

As shown in Figure 2, the saturation concentrations in the assay correspond to 0.25 mg/l for HMG1 and 0.1 mg/l for HMG17. These concentrations represent 50 and 20 ng of protein per well, respectively. The sensitivity of the method allows the detection of 2 ng of HMG/well, using an anti-HMG antibody diluted 1/100.

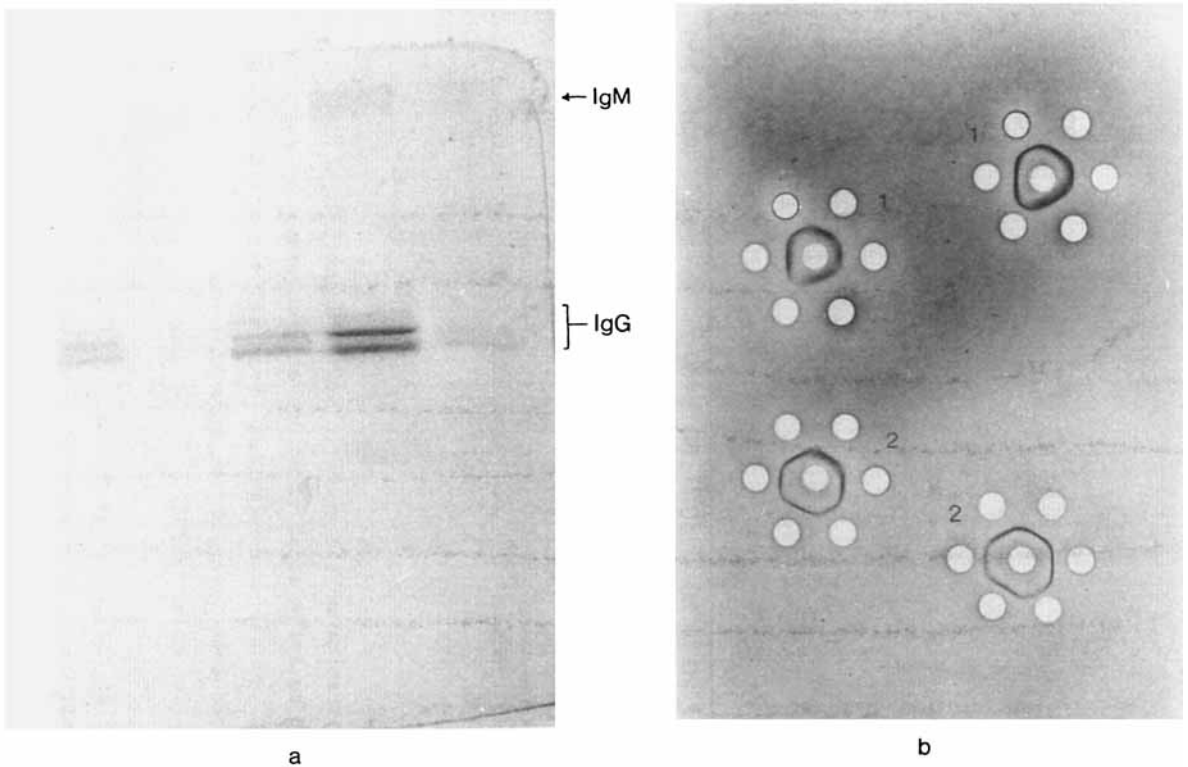

Figure 1. Analysis of the anti-HMG specific antibody solutions purified by affinity chromatography, by two different methods: (a) By PAGE-SDS in 3-20% acrylamide gradient; several samples of anti-HMG1 and anti-HMG17 are shown. (b) By immunodiffusion in agarose gels; central wells contain standard goat anti-rabbit IgG and outer wells contain several anti-HMG1 (1) and anti-HMG17 (2) antibody samples.

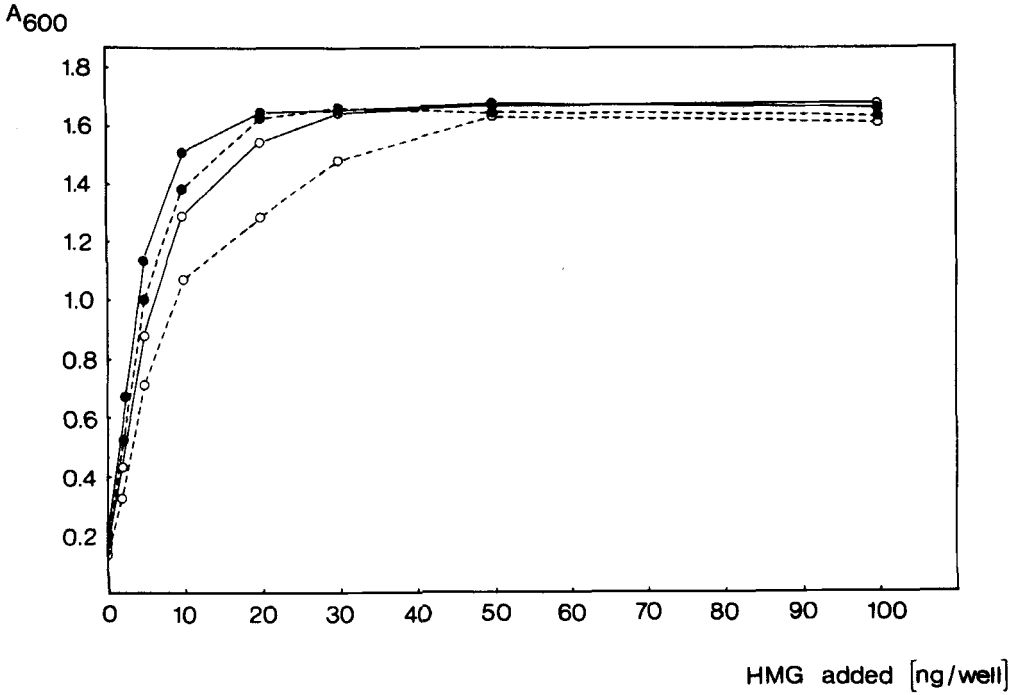

Figure 2. Enzyme immunoassay of a range of HMG1 and HMG17 concentrations versus dilutions 1/100 (——) and 1/500 (— —) of specific anti-HMG1 (O) and anti-HMG17 (●) antibody samples, respectively.

The titration of the specific anti-HMG antibody solutions was carried out assaying eight dilutions of each sample of antibody at the optimal antigen concentration. From all the antibody samples analyzed, the best titer for an anti-HMG1 was 1/4000, and greater than 1/8000 for an anti-HMG17.

Measurement of the Cross-reactivity

To demonstrate the absence of reactivity between the specific anti-HMG antibodies and the heterologous antigen (anti-HMG1 against HMG17 and vice versa) an HMG1 + HMG17 antigen coated

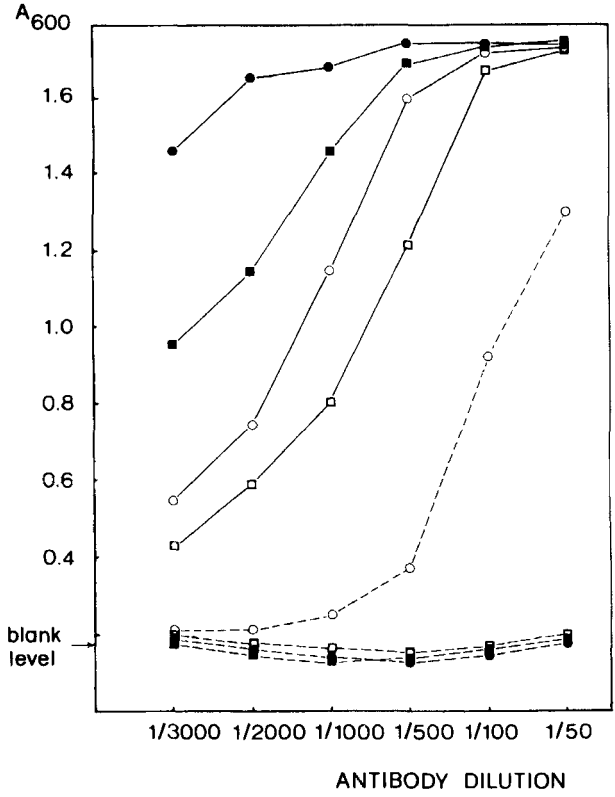

Figure 3. Enzyme immunoassay of two anti-HMG1 (O , ●) and two anti-HMG17 (□ , ■) samples against the homologous (——) and the heterologous (— —) antigen.

microplate was tested by ELISA with several samples of anti-HMG1 and anti-HMG17. As shown in Figure 3, most of the antibody samples only gave positive results when reacted with the homologous antigen. In a single case, an anti-HMG1 antibody showed a measurable titer against the heterologous antigen: 1/100, a concentration much higher than the required to obtain the titer against the homologous antigen, 1/1500.

Figure 4 shows the results of the immunoblotting procedure used to measure the reactivity of the antibodies. Proteins HMG1, 2

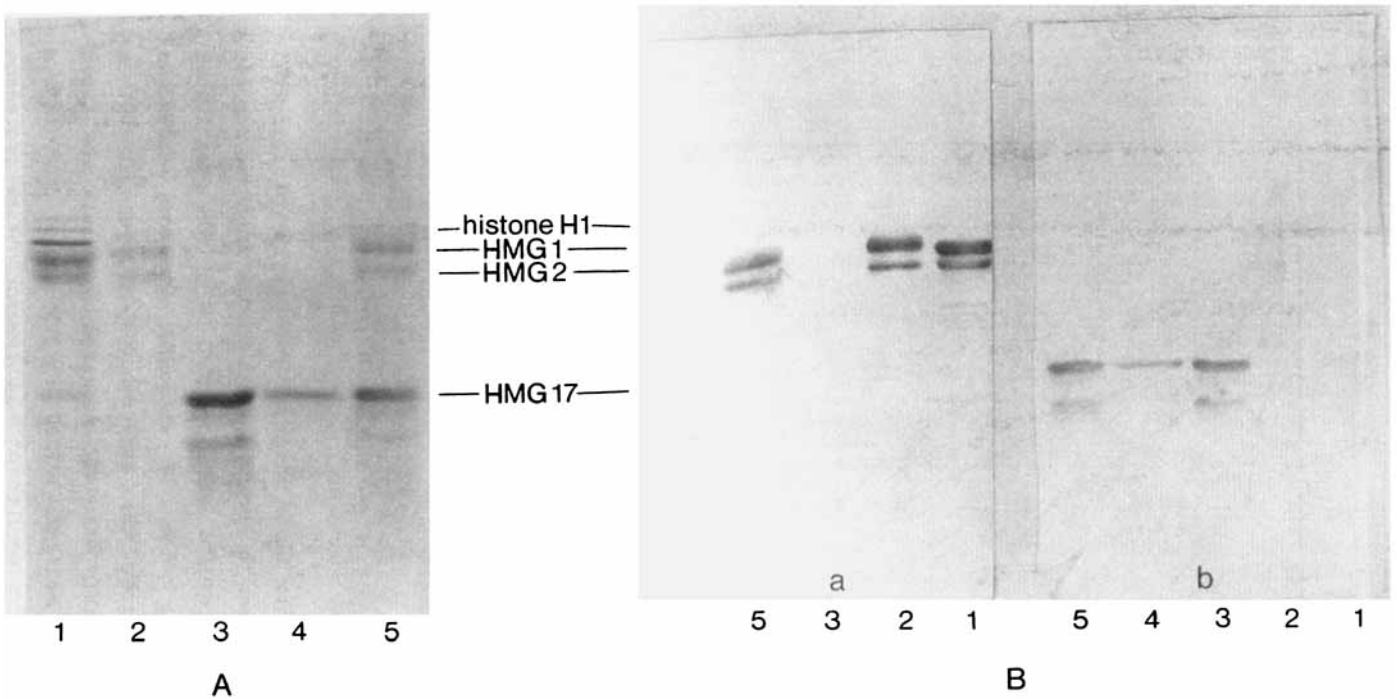

Figure 4. Immunoblotting of the anti-HMG antibody samples. (A) 15% acrylamide gel electrophoresis of calf thymus histone H1 and HMG proteins; (B) immunodetection on nitrocellulose membranes with (a) anti-HMG1 diluted 1/1000 and (b) anti-HMG17 diluted 1/2000. The numbers below the gel and the nitrocellulose membranes indicate the different samples analyzed.

and 17 and histone H1 were loaded into a PAGE-SDS. After their migration, they were transferred to nitrocellulose membranes. At a 1/1000 dilution of the anti-HMG1 antibody (titer by ELISA, 1/4000), only the bands corresponding to HMG1 and HMG2 were revealed. The anti-HMG17 antibody (titer by ELISA, >1/8000) diluted 1/2000 gave positive results only with the HMG17 bands and its degradative fragments.

Detection of HMG Proteins in Native Chromatin

To measure the reactivity of the anti-HMG antibodies with the HMG proteins exposed in native chromatin, an ELISA was carried out using the samples preincubated with digested rat liver chromatin as first antibody. As long as HMG1 and HMG17 are accesible in native chromatin, antibodies will bind to them during the preincubation, and the antibody responses against the antigens coating the plates will decrease. This inhibitory effect can be used to measure the degree of exposure of HMG proteins in native chromatin, and their quantity in discrete-sized fragments of oligonucleosomes.

Figure 5 shows the results obtained in the inhibition assay using a dilution 1/500 of the anti-HMG1 antibody sample, preincubated with 0, 0.5, 1 and 5 mg/l of chromatin digests, and a dilution 1/1000 of the anti-HMG17 antibody sample (a more diluted sample due to its higher titer), preincubated with the same chromatin concentrations. The inhibitory effect of chromatin increases with its concentration, and it is higher for HMG1 protein, revealing its higher degree of exposure in native chromatin.

DISCUSSION

Horseradish peroxidase (HRP) has been widely used in most enzyme immunoassays, but different substrates for this enzyme give different results, in terms of sensitivity and reproducibility

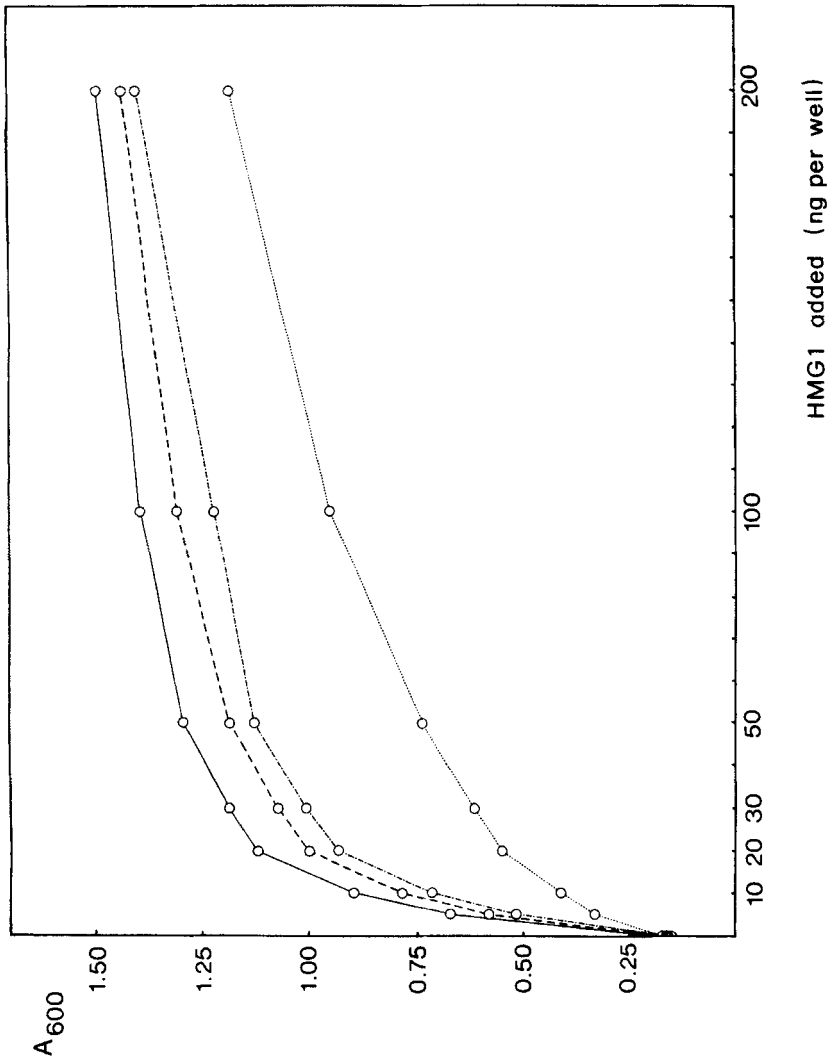

Figure 5

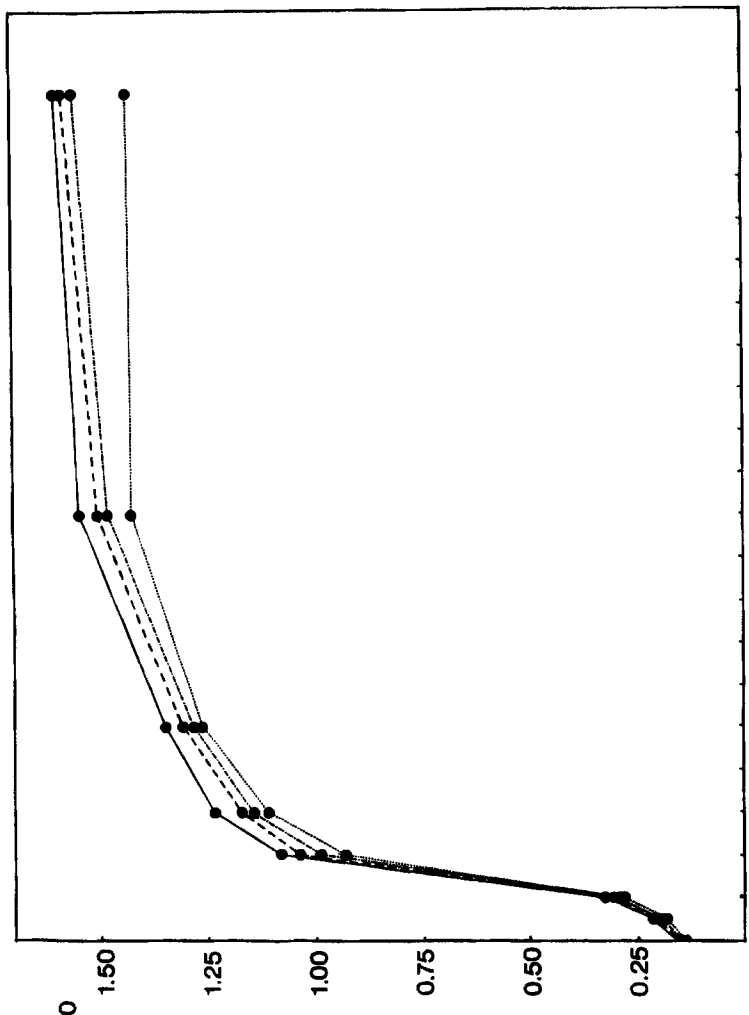

Figure 5. Inhibition enzyme immunoassay with anti-HMG1 (○) and anti-HMG17 (●) antibody solutions diluted 1/500 and 1/1000 respectively, preincubated with 0 (——), 0.5 (– – –), 1 (–·–·–) and 5 (·····) mg/l of chromatin digest.

(14). A very sensitive and versatile nontoxic chromogenic system for peroxidase has been described by Ngo and Lenhoff (6), in which the coupling of MBTH and DMAB in the presence of H_2O_2, catalyzed by HRP, results in a deep-purple compound with an absorbance peak at 590 nm. The high extinction coefficient of the reaction product makes it possible to detect very low concentrations of peroxidase.

In order to determine the optimal concentration of protein bound we studied the adsorption conditions of the assay. Several methods have been used for this determination, among them, the peroxidase saturation of the remaining sites (non occupied by the antigen) of the polystyrene surface (15), measurements with radiolabelled proteins (7,16), and board chequer titration using low affinity sera (15). The use of higher amounts of protein may create artifactual results.

Many immunochemical techniques have been applied to study chromatin structure and organization, by measuring the accesibility of the antigenic determinants of chromosomal proteins (histones and nonhistones) (17). Of importance is the specificity of the antibodies as well as the sensitivity of the immunoassay. In our work, homogeneous populations of specific anti-HMG1 and anti-HMG17 IgG were prepared by affinity chromatography. A titration of different anti-HMG antibody samples was performed, and the crossreactivity between both proteins was tested.

Enzyme immunoassays are easy to perform, safe, and allow to use a wide range of environmental conditions. The ELISA method described here has a detection limit of less than 2 ng protein per well (10 µg/l), as it is shown in Figure 2, similar to that described for RIA (4), but higher than a protein quantitation method by competitive binding on polystyrene beads (18). A different enzyme-immunoassay previously described, using antisera against chicken erythrocyte histones, detected 7.5 ng per well (5). The purified antibodies raised against HMG1 and HMG17 showed no crossreactivity with heterologous protein, in contrast to other reports (2, 11). This allows to distinguish between the antigenic sites of HMG1 and HMG17 packed in nucleosomes and exposed to the binding with specific antibodies.

The accesibility of HMG antigenic determinants is detected by means of an immunoassay with the same antigen coating the plates. The best results were obtained when the antibodies were preincubated with chromatin and the inhibitory effect of this preincubation was measured, rather than binding directly the antibodies upon a chromatin coated plate (results not shown). A good correlation between the increase in chromatin concentration and the decrease in the response of the antibodies to their plate-coated antigens was observed. A clear difference was observed in the accesibility of HMG1 and HMG17 antigenic determinants. The comparative inhibitory activity of chromatin reacting with anti-HMG1 is greater than that with anti-HMG17. This is in agreement with the distribution of HMG1 and HMG17 in nucleosomal chromatin (19, 20), where there is a major accesibility of HMG1, interacting with the linker DNA.

In conclusion, the solid-phase enzyme-linked immunoassay described is a suitable technique for the study of the organization of chromatin with a minimal disruption of its native structure, by quantitative binding of specific antibodies elicited to the different chromosomal components.

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