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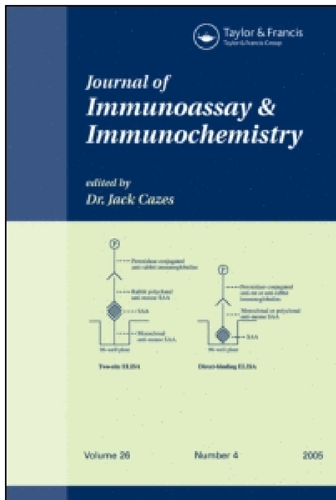

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Sang Ho Kweon[a]; Tatsuzo Oka[a]; Mikiko Ito[a]; Masao Morita[a]; Yasuo Natori[a]
[a] Department of Nutritional Chemistry, School of Medicine, The University of Tokushima Kuramoto, Tokushima, Japan

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MONOCLONAL-ANTIBODY-BASED ENZYME-LINKED IMMUNOSORBENT ASSAY FOR HMG 2b NONHISTONE PROTEIN IN CHICK LIVER

Sang Ho Kweon, Tatsuzo Oka, Mikiko Ito, Masao Morita
and Yasuo Natori
Department of Nutritional Chemistry, School of Medicine, The University of Tokushima
Kuramoto, Tokushima 770, Japan

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ABSTRACT

Monoclonal antibodies were prepared against the high mobility group (HMG) protein 2b from chick liver chromatin and a monoclonal-antibody-based enzyme-linked immunosorbent assay (ELISA) was developed for chick HMG 2b. The sensitivity of the assay is about ten times that of the previously described radioimmunoassay and solid-phase enzyme immunoassay for HMG proteins. With the use of ELISA technique, the amount of HMG 2b (μg protein/mg DNA) in the livers of 1-day old and 70-day old chicks was found to be 2.56 ± 0.4, and 1.20 ± 0.2, respectively. The age-dependent change in the level of HMG proteins probably reflects changes in the functional state of chromatin during ageing.

(KEY WORDS: High mobility group protein, Chick liver, Monoclonal-antibody-based ELISA)

ADDRESS: All correspondence should be sent to Dr. Yasuo Natori, Department of Nutritional Chemistry, School of Medicine, The University of Tokushima, Kuramoto, Tokushima 770, Japan.

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INTRODUCTION

High mobility group (HMG) proteins are a family of non-histone components in chromatin of relatively low molecular weights and are thought to be involved in the structure of transcriptionally active chromatin (1,2). There are four major HMG proteins (HMG 1, 2, 14 and 17) in all the eukaryotic cells examined to date (3). In chicks, HMG 2 has been further resolved by chromatography into two subfractions, called HMG 2a and 2b (4).

Previous work in our laboratory has shown that HMG proteins play an important role in nutritional modulations of chick liver RNA synthesis (5). To understand the functions of HMG proteins, it is essential to determine the cellular levels of these proteins under various physiological conditions. The aim of this paper is to describe a monoclonal-antibody-based enzyme-linked immunosorbent assay for chick HMG 2b. Comparison of HMG 2b contents in newly hatched and adult chicks indicates an age-dependent decrease in liver HMG 2b contents.

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MATERIALS AND METHODS

Animals

Newly hatched and 70-day old male White Leghorn chicks were obtained from a local hatchery (Ishii Hatchery, Tokushima).

Preparation of Liver Samples

For the quantification of HMG 2b protein in the liver, the livers were homogenized in 2.5 volumes of cold 0.25 M sucrose in buffer A [50 mM Tris-HCl (pH6.5), 25 mM KCl, 1 mM phenylmethyl sulfonyl fluoride and 10 mM $MgCl_2$] with a Potter-Elvehjem-type homogenizer. After dilution with an equal volume of the homogenizing medium, the homogenate was further treated with a Polytron homogenizer (Kinematica GmbH, Kriens/ Luzern, Switzerland). The homogenate was then centrifuged at 3,000 rpm for 5 min, and the resultant supernatant was used as the sample for HMG 2b assay.

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Preparation of HMG 2 from Chick Liver

The total HMG proteins were extracted from 70 day-old chick livers by the procedure of Seyedin and Kistler (6). The isolation of HMG 2 and the resolution of HMG 2a and 2b were performed as described by Mathew et al. (4).

Preparation of Antiserum

Male New Zealand white rabbits were immunized with HMG 2 (HMG 2a and 2b, unresolved) dissolved in 10 mM Tris-HCl buffer (pH7.4) and emulsified in complete Freund's adjuvant (1:1 v/v). Each animal received subcutaneous injections on days 1, 7 and 15; the dose was 0.1 mg of HMG 2 in a total volume of emulsion of 0.5 ml. Booster injections of 0.05 mg HMG 2 in the emulsion of 50% Freund's incomplete adjuvant were given 1 week later. The animals were bled weekly after the booster injection.

Preparation of Monoclonal Antibodies

BALB/c mice were injected intramuscularly in the haunch with 0.1 mg HMG 2 in 0.2 ml of the emulsion of 50 % complete Freund's adjuvant on day 1, 14 and 30. One month after the third injection, animals received a single injection of HMG 2 (0.01 mg in 0.2 ml saline) via the tail vein. After 3 days, spleen cells from the immunized mice were fused with SP/2 myeloma cells. Hybridoma cell propagation, subcloning and ascites amplification procedures were performed as described by Vanderbilt and Anderson (7). The isotype of the monoclonal antibody against HMG 2 was determined by the use of mouse monoclonal antibody isotyping kit (Amersham, UK).

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Immunoblotting

Ten µg of HMG 2a and 2b, either individually or in combination, were dotted to nitrocellulose membranes and the membranes were blocked with 3% gelatin in the washing buffer (PBS containing 0.05% Tween 20) for 30 min at room temperature. The polyclonal antibody against HMG 2 (diluted IgG fraction containing 8 µg protein/ml) or monoclonal antibody against HMG 2b (diluted ascites containing 6 µg protein/ml) was added to the nitrocellulose membranes, and incubated for 2 h. The membranes ware washed three times with the washing buffer. After washing, horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgM (Cappel Products, USA) diluted 1/3000 in PBS was added to the membranes and incubated at room temperature for 40 min.

The membranes were washed three times with the washing buffer and developed by addition of 3,3'-diaminobenzidine and H_2O_2.

Monoclonal-antibody-based enzyme-linked immunosorbent assay (ELISA)

An ELISA plate (96-well, Sumitomo Bakelite Co., Tokyo) was precoated with monoclonal antibody against HMG 2b (diluted ascites containing 6 μg protein/ml) The wells were washed three times with the washing buffer (PBS containing 0.05% Tween 20) and blocked with 1% skim milk powder in PBS for 1 h. Samples, containing up to 20 ng of HMG 2b, were added to each well and incubated for 4 h at 37℃. The wells were washed three times with the washing buffer. After washing, polyclonal antibody against HMG 2 (diluted IgG fraction containing 8 μg protein/ml) was added and incubated for 1 h at 37℃. After washing the wells three times with the washing buffer, horseradish peroxidase-conjugated goat anti-rabbit IgG(1/3000 dilution) were added to each well and incubated at room temperature for 1 h. The substrates, 2,2'-azinobis(3-ethylbenzthiazole sulfonic acid) and H_2O_2, were added to each well. After 15-30 min, the absorbancy at 415 nm was measured with an automated microplate reader (Corona Co., Tokyo).

Protein and DNA Measurements

Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as the standard. DNA was determined by the diphenylamine method of Burton (9).

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RESULTS

Characterization of Monoclonal Antibodies

Hybridomas producing monoclonal antibodies against HMG protein were generated by immunizing mice with a mixture of HMG 2a and 2b. Two cell lines that produced antibodies reacting with HMG 2 were recovered and the antibody possessing the stronger reactivity was used for the present study. The antibody was found to be the IgM type. Immunoblotting analysis of HMG 2 was performed under denaturing and non-denaturing conditions. As seen in Fig. 1-A, the antibody was reactive with the native HMG 2 but failed to react with SDS-treated HMG 2 after protein-blotting procedures. The determinants recognized by the antibody presumably required the native protein conformation for the antigenic integrities. The differential reactivities of the antibody with HMG 2a and 2b showed that the antibody specifically recognized HMG 2b (Fig. 1-B).

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ELISA of HMG 2b

Monoclonal-antibody-based ELISA was carried out as described under MATERIALS AND METHODS. We observed that the binding of HMG 2b to plates precoated with monoclonal antibody against HMG 2b occurred at rather unusual pH and salt concentrations. As shown in Fig. 2, the binding was maximal at pH 5.2 and decreased sharply at more acidic or more basic pHs. The binding was also sensitive to the salt concentrations; the concentration of 250 mM NaCl gave the maximal binding (Fig. 3)

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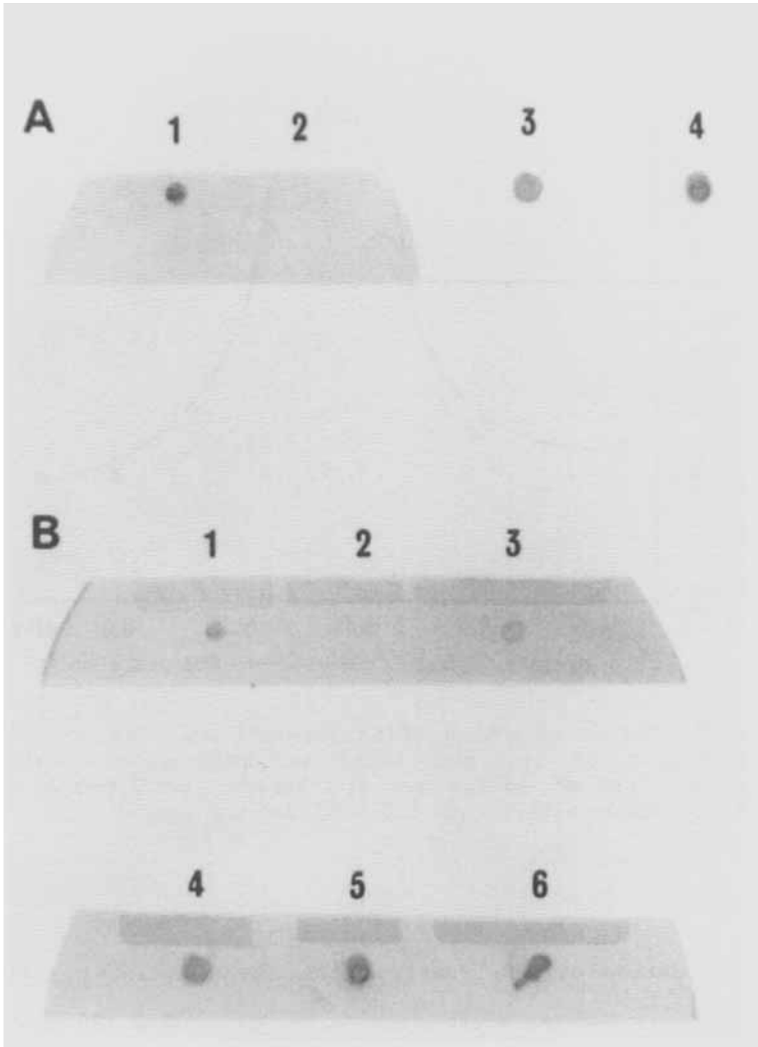

Figure 1. Immunostaining of HMG 2 by monoclonal and polyclonal antibodies. A. Native (1, 3) or SDS-treated (2, 4) HMG 2 was dotted to nitrocellulose membranes and incubated with the monoclonal (1, 2) or polyclonal (3, 4) antibodies. For the SDS-treatment, samples in 2.3% SDS/1% mercaptoethanol/10 mM Tris-HCl (pH 6.8) were heated for 2 min in boiling water. B. Native HMG 2b (1, 4), HMG 2a (2, 5), and HMG 2a + 2b (3, 6) were dotted to nitrocellulose membranes and incubated with the monoclonal (1, 2, 3) or polyclonal (4, 5, 6) antibodies.

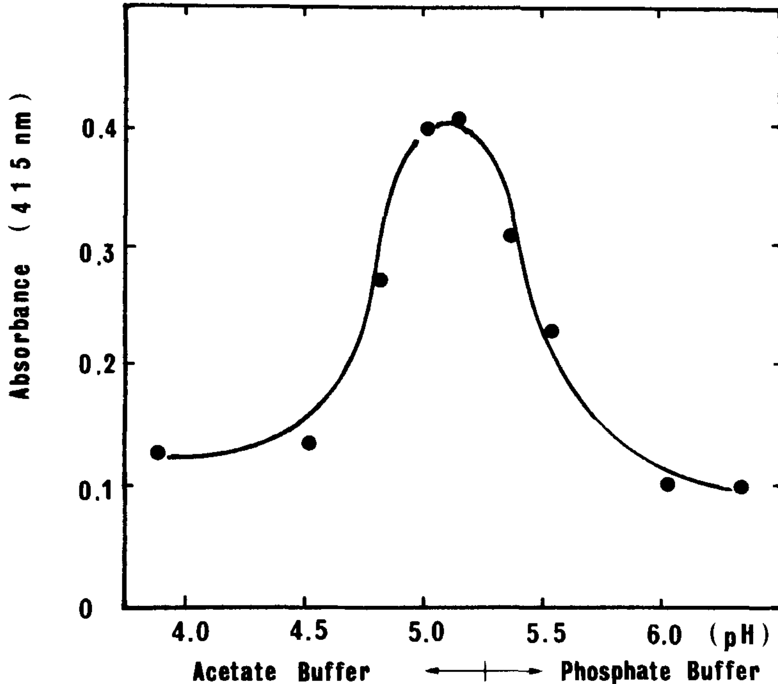

Figure 2. Effect of pH on ELISA for HMG 2b. Purified HMG 2b was incubated in the monoclonal antibody-coated plates at various pHs (50 mM sodium acetate buffer, pH 3.8-5.3; 50 mM sodium phosphate buffer, pH 5.5-6.3) in the presence of 250 mM NaCl.

The incubation of the samples with the monoclonal antibody-coated plates was therefore carried out at pH 5.2 (50 mM sodium acetate buffer) and 250 mM NaCl. A typical calibration curve of ELISA using purified HMG 2b is shown in Fig. 4. The curve was essentially linear up to 15 ng HMG 2b. The assay was specific to HMG 2b; HMG 1 or histone 1 did not produce any color development. The coefficients of intra-assay variations were 4.9 - 9.5% (n = 10).

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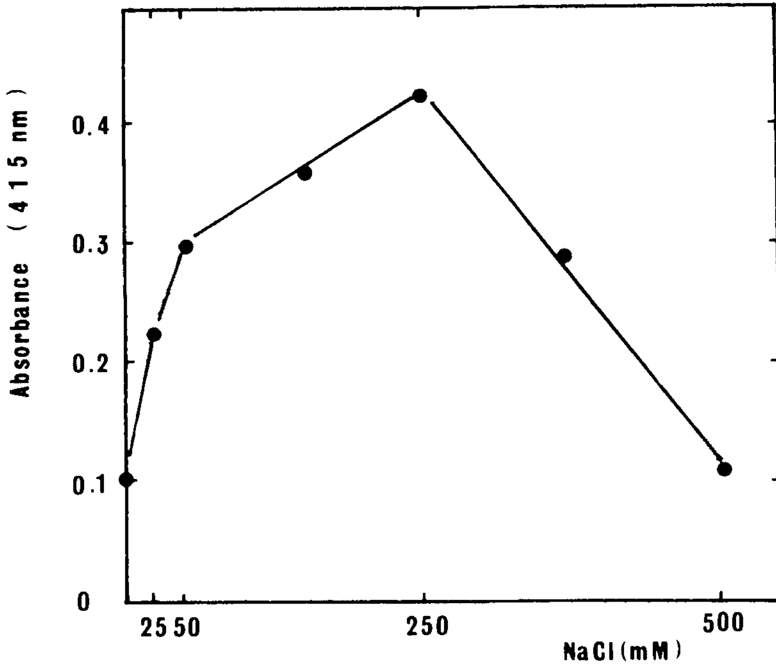

Figure 3. Effect of NaCl concentrations on ELISA for HMG 2b. Purified HMG 2b was incubated in the monoclonal antibody-coated plates at pH 5.2, containing increasing concentrations of NaCl.

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Quantification of HMG 2b in Newly Hatched and Adult Chick Livers

Utilizing the presently developed ELISA, we determined the amounts of HMG 2b in the homogenates of the livers from newly hatched (1-day old) and adult (70-day old) chicks. The HMG 2b contents were 2.56 ± 0.4 μg and 1.20 ± 0.2μg/mg DNA for 1-day old and 70-day old chick livers, respectively. The values represent means ± S.D. for 5 animals in each group. It thus

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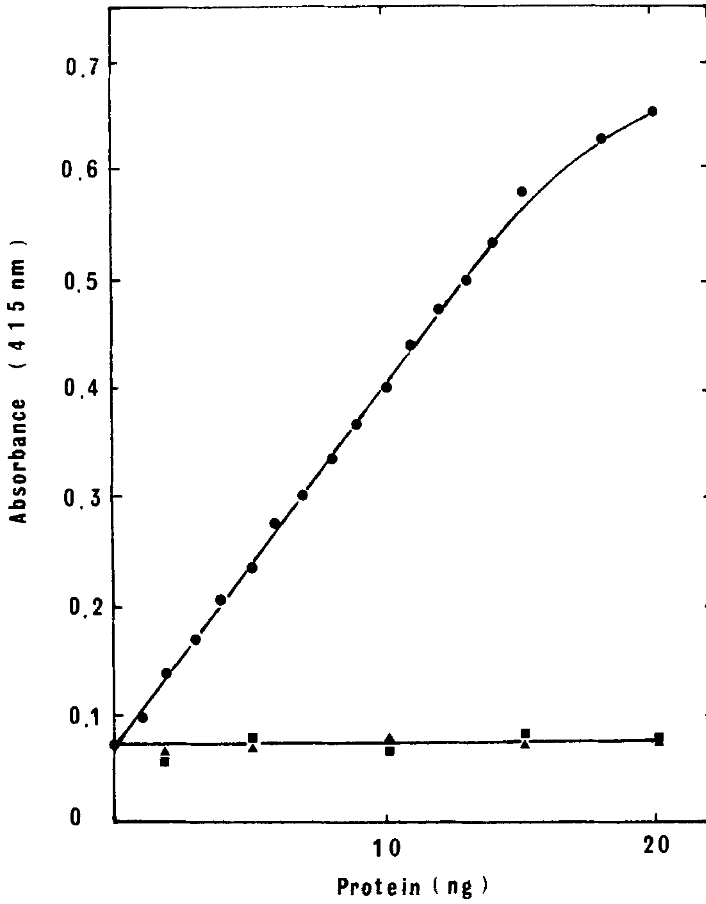

Figure 4. Calibration curve of ELISA for HMG 2b. HMG 2b (●), HMG 1 (■) and histone 1 (▲).

appears that the HMG 2b content in the liver of chicks decreases with ageing.

DISCUSSION

Preparation of monoclonal antibodies against HMG 1, 2a and 2b from hen erythrocyte chromatin has been reported by Vanderbilt and Anderson (7) but ELISA, utilizing these antibodies has not been described. In this communication the adaptation of monoclonal-antibody-based ELISA for HMG 2b is described. The technique is at least 10 times more sensitive than the previously described radioimmunoassay (10, 11) and the range of linearity of the calibration curve is twice that of the solid-phase enzyme immunoassay (12) for HMG 1 protein.

With the use of our ELISA technique, the amount of HMG 2b (μg protein/mg DNA) in the livers of 1-day old and 70-day old chicks was found to be 2.56 ± 0.4 and 1.20 ± 0.2, respectively. These values are comparable to the reported values of 1-5μg HMG 2/mg DNA for the rat somatic tissues (13).

Regarding the age-related change of HMG protein levels, Prasad and Thakur (14) recently reported that the HMG 2 level essentially remained unchanged in the livers of young (19-week old) and old (118-week old) rats. However, we have previously obtained data showing that the HMG 2 level in the liver of 2-week old rats is twice that of 10-week old rats (15). The age-

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dependent change in the level of HMG proteins probably reflects changes in the functional state of chromatin during ageing. Further works are in progress to elucidate the molecular mechanism of HMG protein metabolism.

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