Detection of monoclonal antibody to high-mobility-group protein 17 from chick oviduct

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Total chromosomal HMG (high-mobility-group) proteins have been isolated from oestrogen-stimulated chick oviduct. The antibodies against these proteins were induced in mice and subsequently their spleen cells were fused with myeloma cells to form hybridomas. A highly purified HMG protein, 17, was used to select for the hybridomas that produce antibody against HMG protein 17. The hybridomas were cultured and injected into mice to produce ascites. The antibody against HMG protein 17 in the IgG (immunoglobulin G) fraction of the ascites fluid was obtained by Protein A–Sepharose column chromatography. We have devised a solid-phase radioimmunoassay and enzyme-linked serological assay for the detection and characterization of this antibody directed against HMG protein 17. This anti-(HMG protein 17) IgG interacted only with HMG protein 17, but not with other chromosomal proteins, e.g. histone H1, '95K protein' (a chick oviduct-specific chromosomal protein) and HMG proteins 1, 2 and 14. The monospecific nature of this anti-(HMG protein 17) IgG fraction is confirmed.

Chromosomal non-histone high-mobility-group (HMG) proteins are universally present in the cells of various organisms (Goodwin & Johns, 1973; Alfageme *et al.*, 1976; Franco *et al.*, 1977; Watson *et al.*, 1977; Rabbani *et al.*, 1978; Spiker *et al.*, 1978). They consist of four major proteins: HMG-1, -2, -14 and -17. We first reported the isolation, purification, characterization and hormone responsiveness of the HMG proteins in the chick oviduct (Teng *et al.*, 1979; Teng & Teng, 1981). Similar observations comparable with ours have been reported in calf thymus, liver and chick erythrocytes (Goodwin *et al.*, 1975; Vidali *et al.*, 1977; Levy *et al.*, 1977; Sterner *et al.*, 1978; Goodwin & Johns, 1978).

The DNA associated with the nucleosomes containing HMG-14 and -17 is enriched with active globin gene sequences (Albanese & Weintraub, 1980). The major sites of interaction of these two proteins are located in the ends of the nucleosome core DNA (Sandeem *et al.*, 1980; Mardian *et al.*, 1980). The importance of these two HMG proteins in association with active transcribed nucleosomes has been revealed by the finding that they enhance the chromosomal sensitivity to digestion by DNA-

ase (deoxyribonuclease) (Weisbrod & Weintraub, 1979; Weisbrod *et al.*, 1980).

Workers in our laboratories are interested in investigating the changes in quantity and quality of HMG proteins during oestrogen stimulation in the chick oviduct. Initially, we have applied immunobiochemical techniques to investigate the localization and quantity change of HMG-1 in chick oviduct cell (Teng & Teng, 1981). This conventional immunoassay technique is dependent on the antibody produced against HMG protein in rabbit. This technique was hindered by the limited specificity of the antibody so induced. Therefore we have tested the possibility of obtaining a monospecific antibody against HMG proteins.

In the present paper we report the purification of HMG protein 17 from oestrogen-induced chick oviduct and applying it for antibody induction by hybridoma cell fusion. The antigenicity of this HMG-17 antibody has been tested with various nuclear proteins by an enzyme-linked antibody assay technique and solid-state radioimmunoassay, and they proved that it is monospecific in nature.

Materials and methods

Animals and chemicals

Fertile White-Leghorn-chicken eggs, obtained from Rich-Glo Farms, Houston, TX, U.S.A., were placed in a humidified incubator (Petersime model 4)

Abbreviations used: HMG protein, high-mobility-group protein; Ig, immunoglobulin.

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at 38°C. The female chicks were individually injected 10 days after birth with 1.25 mg of oestradiol-17 β in 0.25 ml of propylene glycol (propane-1,2-diol) daily for 3 weeks. The chicks were killed by cervical dislocation and the oviducts were removed for nuclear preparation. The total HMG proteins and HMG protein 17 were extracted from the nuclei as previously described (Teng *et al.*, 1979; Sanders & Johns, 1974). The following chemicals were obtained from the sources indicated. Purine, pyrimidine and *O*-nitrophenyl galactoside were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); peroxidase and rabbit anti-mouse IgG were from Miles Laboratories (Elkhart, IN, U.S.A.); Na¹²⁵I was from New England Nuclear Corp. (Boston, MA, U.S.A.); Protein A–Sepharose CL-4B and CM-Sephadex were from Pharmacia Fine Chemicals (Uppsala, Sweden); Dulbecco minimal essential (DME) medium was from Gibco (Grand Island, NY, U.S.A.). All other chemicals were of analytical grade. The plasmacytoma cell line (P3-X63Ag8) was from the Salk Institute (La Jolla, CA, U.S.A.).

Production of mouse anti-(HMG protein) antiser

Total HMG protein purified from chick oviduct was suspended in 0.05 M-Tris/HCl buffer, pH 7.5, at a concentration of 1 mg/ml and emulsified in an equal volume of Freund's complete adjuvant. Three Balb/c mice were injected intraperitoneally with 0.2 ml of the protein suspension and boosted 3 weeks later with identical protein emulsified in Freund's incomplete adjuvant. Antiserum was collected from the retro-orbital plexus 10 days after the last injection and stored at -70°C until use.

Mouse anti-(HMG protein) hybrid cell lines

The immunized Balb/c mice that produced anti-(HMG protein) antiser were boosted with total HMG protein and killed 3 days later. Spleen cells (10⁸ cells) from an immunized mouse were fused with 10⁷ plasmacytoma cell lines. We used the detailed fusion technique described by Kennett *et al.* (1978). The resulting hybrids were suspended in DME medium with 4.5 g of glucose/litre and 0.5 mg/ml (w/v) glutamine and 10% (v/v) foetal-bovine serum. In addition, purine, pyrimidine, thymidine (16 μ M each) and hypoxanthine (0.11 M) were added to the cell culture medium. The fused cells were evenly suspended and distributed into six micro-plates, with one drop (50 μ l) per well. After culture for 24 h, an additional drop (50 μ l) of the medium containing aminopterin (0.8 μ M) was added. In general, the clones appear macroscopically within 2 weeks. The antibody secreted from hybridoma into the medium was detected by an enzyme-linked immunosorbent assay ('ELISA').

Identification of antibody by enzyme-linked immunosorbent assay

The presence of antibody (against HMG proteins) in the medium was probed by procedures modified from those described by Kennett (1980). Total HMG protein (or purified individual proteins HMG-1, -2, -14 and -17) was dissolved in borate buffer at a concentration of 30 μ g/ml and 150 μ l was delivered into the wells of a microtitre plate. The plates were incubated at 37°C for 4 h, then washed with 0.6% Tween/saline solution [0.85% (w/v) NaCl/0.05% (v/v) Tween-20] eight times. The medium (150 μ l) containing antibody was tested by adding it to the HMG-protein-coated well and incubated at room temperature by shaking for 4 h, then washed six times (6 ml each time) with Tween/saline solution. To each well, 150 μ l of β -galactosidase conjugate [diluted to 1:100 with PBS buffer (0.05 M-sodium phosphate/0.14 M-NaCl, pH 7.2), containing 0.05% Tween-20] was added and incubated for 2 h at room temperature. Then the wells were washed with Tween/saline as previously described. Enzymic reaction was started by adding *O*-phenylenediamine solution (1 g/ml, w/v), containing 0.012% H₂O₂ in 0.1 M-citrate buffer, pH 4.5, to a final volume of 0.2 ml. The reaction took place within 30 min and was stopped by adding 5 μ l of 1.0 M-NaF. The intensity of the colour development is proportional to the strength of the antibody produced. The hybridoma secreting a different antibody against each individual HMG protein could be identified. A large quantity of antibody was produced by growing these hybridomas as ascites tumours in Balb/c mice.

Purification of HMG-17 antibody

The ascites fluid containing HMG-17 antibody IgG was obtained by gel filtration through a column of Protein A–Sepharose CL-4B as described by Tucker *et al.* (1978). The column was run at 20 ml/h and the fractions corresponding to HMG-17 antibody IgG were pooled. After dialysis overnight with two changes of PBS buffer, the antibody was precipitated by 50%-saturated (NH₄)₂SO₄ and redissolved in water. The purified antibody was stored at -70°C until use.

Radioiodination of proteins

The immunoglobulin fraction of rabbit anti-mouse IgG (50 μ g) was labelled with 1 mCi of Na¹²⁵I by using immobilized lactoperoxidase beads as described by Newman *et al.* (1981). Unbound ¹²⁵I was removed from the reaction mixture by gel filtration through a 10 ml Sephadex G-15 column equilibrated in PEB buffer [0.05 M-sodium phosphate buffer containing 0.14 M-NaCl, 1 mM-EDTA and 1%

(w/v) bovine serum albumin, pH 7.2]. Fractions containing approx. 98% bound radioactivity were pooled and stored at -80°C . A specific radioactivity of $2\text{--}4\ \mu\text{Ci}/\mu\text{g}$ was routinely obtained.

Radioimmunoassays for detecting anti-(HMG-17) antibody

The assay was performed in $12\text{ mm} \times 75\text{ mm}$ polystyrene test tubes that had been coated with antigen [$10\ \mu\text{g}$ of HMG-17 or other proteins in 0.6 ml of PBS buffer containing 0.1% (w/v) gelatin] overnight. To each tube containing 0.2 ml of diluted ascites, IgG (1:20 dilution in PBS/gelatin buffer) was added and the mixture was allowed to incubate for 2 h at 37°C . The tubes were then washed three times with 0.6 ml of PBS/gelatin buffer followed by the addition of $50\ \mu\text{l}$ of ^{125}I -labelled rabbit anti-mouse IgG containing $150\,000\text{ c.p.m.}$ of radioactivity. After a 2 h incubation at room temperature, the tubes were washed three times in PBS/gelatin buffer, and the radioactivity of the tube was measured with a Traco 1191 gamma counter (counting efficiency 83%).

Gel electrophoresis of HMG protein

Total HMG proteins or purified HMG-17 were separated by acetic acid/urea/polyacrylamide-gel electrophoresis as described by Panyim & Chalkley (1969) and as modified by Teng *et al.* (1979).

Results and discussion

We have applied the standard HClO_4 extraction method for the isolation of total HMG proteins and then resolved them by polyacrylamide-gel electrophoresis. The total HMG proteins from chick oviduct and calf thymus were compared, and they show a striking difference in proportion between the individual proteins (Figs. 1a and 1b). In calf thymus, HMG-1 and -2 are more prominent than those of the chick oviduct. However, HMG-14 and -17 are much more abundant in the oviduct. The higher concentration of HMG-14 and -17 in oviduct probably reflects the physiological state of the organ, since the proteins are extracted from chick oviducts that were extensively stimulated with oestrogenic hormone for 3 weeks. In this hormonal environment the cellular proliferation and the organ-specific genomes (i.e. ovalbumin, conalbumin and lysozymes) in chick oviduct are greatly induced. Therefore the concentration of these HMG proteins is expected to increase in correspondence to the hormonal action.

The increase in HMG-protein synthesis in co-ordination with cellular proliferation and genomic activation has been demonstrated in rat testis, chicken skeletal muscle and rat liver cell during regeneration (Seyedin & Kistle, 1979; Huehl, 1979). Moreover, we have demonstrated that, in chick

Fig. 1. Polyacrylamide-gel electrophoresis of HMG proteins

(a) Total HMG proteins extracted from calf thymus nuclei. (b) HClO_4 -soluble protein (from chick oviduct nuclei) precipitated with 6 vol. of acetone (H1 = histone H1). (c) Total HMG protein from oviduct nuclei after removal of histone H1. Gel electrophoresis with 15% (w/v)-polyacrylamide gel, 0.1% NN' -methylenebisacrylamide, 0.9 M -acetic acid, 2.5 M -urea, 0.125% $NNN'N'$ -tetramethyl-phenylenediamine and 0.125% ammonium persulphate was performed as described by Teng *et al.* (1979). The numbers on the right side of gel (c) represent HMG-1, -2, -3, -14 and -17. The protein bands -18 and -19 are the newly discovered HMG proteins.

oviduct, HMG-1 on a per-single-cell basis is greatly induced after 5 days of oestrogen stimulation. The quantity is increased 4-fold in the cytosol, 3.5-fold in the microsomal fraction and 1.6-fold in the nuclear fraction (Teng & Teng, 1981). In comparison with the total HMG proteins isolated from immature chick oviduct, the amounts of four HMG proteins are higher in oestrogen-stimulated oviduct cells (results not shown).

According to the fractional-acetone-precipitation method developed by Rabbani *et al.* (1978), histone H1 could be removed from the total-HMG-protein preparation. Gel electrophoresis indicated that, after histone removal, the total-HMG-protein preparation consists of four major HMG proteins, namely HMG-1, -2, -14 and -17 (Fig. 1c). In addition, two

distinct protein bands with higher electrophoretic mobility than HMG-17 were consistently observed in the present (Figs. 1b and 1c) and in our previous (Teng & Teng, 1981) study. We have tentatively designated them as HMG-18 and -19. A similar observation has been made by Goodwin *et al.* (1981), that, in chicken erythrocyte and oviduct, two 'HMGY'-protein bands that were associated with the active nucleosomes have been discovered. The isolation and further characterization of these two proteins is necessary. Total HMG proteins were administered to mice for antibody induction. We expected to gain at least one antibody (or more than one) against each HMG protein. Subsequently we were able to use an individual purified HMG protein to select the hybridoma clones for individual culture and antibody production.

Because of the experimental design, it is important to obtain a pure HMG-protein preparation for this purpose. The CM-Sephadex column-chromatographic separation for chick oviduct HMG proteins is modified from that described in our previous publication to obtain HMG-1, -2, -14 and -17 (Teng *et al.*, 1979). In general, HMG-14 and -17 tend to aggregate to each other; therefore selective extraction with the 18% (w/v)-trichloroacetic acid method described by Goodwin *et al.* (1978) provided a way of separation for these two proteins. It is therefore possible to obtain pure HMG-17 (Figs. 2e and 2f). These two proteins have a very similar amino acid composition in comparison with their counterparts from calf thymus, chick erythrocytes and trout

testis, with low concentrations of cysteine, methionine, isoleucine, leucine, threonine and phenylalanine (Wigle & Dixon, 1971; Goodwin *et al.*, 1978; Walker *et al.*, 1980). The basic amino acids, i.e. lysine and arginine, are generally present in high concentration (Table 1). The similarity of HMG-14 and -17 between different species and organisms indicates they are evolutionarily conserved.

When the mice received total HMG protein administration for 3 weeks, the antibodies against HMG proteins were induced and detectable in blood serum by an immunodiffusion plate (results not shown). The spleen cells from one of these mice were isolated and fused with plasmacytoma cells and the resulting hybridoma cells were cultured. Subsequently the culture medium containing the secretion of the hybridoma cells was tested for the presence of antibody. The enzyme-linked antibody assay ('ELISA') was performed and 26 positive clones for total HMG proteins were identified. Among these clones, four belong to HMG-17 (results not shown). One of the specific clones for HMG-17 has been recloned to obtain a large quantity of hybridoma cells. These cells were then injected into mice to produce anti-(HMG-17) antibody in ascites- and solid-tumour forms. Consistently, within 2 weeks, 6 ml of ascites fluid is produced from one mouse.

To ensure that the antibody produced in the mouse ascites is in the IgG form, we passed the

Fig. 2. Purification of HMG-17 protein (a) and (b) are total HMG proteins from chick oviduct and calf thymus respectively. (c) is the crude HMG-1 and -2 preparation. (d)–(f) are different preparations of HMG-17 protein. Abbreviation used: H1, histone H1.

Table 1. Amino acid composition of chick oviduct HMG-14 and -17 proteins

The protein (150 µg) was hydrolysed with acid and analysed for amino acid content on a Beckman model 121 amino acid analyser as described by Spackman *et al.* (1958).

Amino acid	Content (mol/100 mol)	
	HMG-14	HMG-17
Asp	9.6	9.4
Thr	3.0	2.2
Ser	4.5	2.5
Glu	12.8	10.5
Pro	12.2	12.9
Gly	10.7	11.2
Ala	16.3	18.4
Val	3.0	2.5
Cys	—	—
Met	0.1	—
Ile	0.2	—
Leu	1.5	—
Try	0.1	—
Phe	0.1	Trace
Lys	20.9	24.3
His	0.2	—
Arg	4.8	4.1
Acidic/basic	0.86	0.71

ascites fluid through a Protein A–Sepharose column (results not shown). The antibody eluted at pH 8 represents the IgA and IgM fractions, whereas that eluted at pH 3 represents the IgG fraction. Two experimental designs were used to detect the monospecificity of this IgG fraction. First, enzyme-linked immunosorbent assays indicated that the ascites fluid and the IgG fraction consisted of a strong antibody activity against HMG-17. A significant amount of activity was detected in the tumour extract and only a trace amount of activity was found in the mouse serum and the IgA-plus-IgM fraction. There was no detectable activity in the reaction buffer (Table 2). This indicated that the HMG-17 antibody resides in the IgG form. Secondly, a solid-phase radioimmunoassay has been applied to test the specificity of the IgG fraction. Results presented in Table 3 confirmed the previous observation that the IgG fraction of the ascites fluid consists of the HMG-17 antibody that is monospecific to its own antigen. Other proteins, e.g. histone H1, '95K protein' (a chick oviduct-specific chromosomal protein) and HMG-1, -2, and -14 do not interact with HMG-17 antibody.

Table 2. *Testing the antigenicity of HMG-17 antibody by enzyme-linked serological assay*

The techniques for antibody assay are described in the Materials and methods section. The second antibody was linked to β -galactosidase (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) and *O*-nitrophenyl galactoside (1 mg/ml) was used as the substrate. The protein concentration in the IgG or the IgA + IgM fraction was 150 μ g/ml, and in other fractions, 1.0 mg/ml. Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard. The antigenic reaction was determined by reading the A_{405} with an Artek automatic ELISA reader, model 210. The symbols representing the range of A_{405} are defined as follows: —, from 0 to 0.08; +, from 0.08 to 0.2; ++, from 0.2 to 0.5; +++, over 0.5 unit.

Source of antibody	Antibody dilution (fold)	Antigen (HMG-17) reaction
Mouse serum	100	+
	1000	—
Ascites fluid	100	+++
	1000	++
Tumour extract	100	++
	1000	—
IgG fraction	100	+++
	1000	++
IgA + IgM fraction	100	+
	1000	—
PBS buffer		—

Table 3. *Detection of HMG-17 antibody monospecificity*

A solid-phase radioimmunoassay was used to detect the monospecificity of the IgG fraction of HMG-17 antibody obtained from the Protein A–Sepharose column. The details of this assay were described in the Materials and methods section. The values presented are means $\pm$ S.D. of three to four determinations.

Protein serving as antigen	125 I-labelled anti-mouse IgG bound (c.p.m.)
Buffer only	523 $\pm$ 19
Buffer + antibody (IgG)	808 $\pm$ 25
HMG-17	2450 $\pm$ 45
Histone H1	862 $\pm$ 23
'95K protein' (chick oviduct)	841 $\pm$ 24
HMG-1	539 $\pm$ 20
HMG-2	536 $\pm$ 16
HMG-14	760 $\pm$ 22

In conclusion, the adaptation of monoclonal antibody and solid-phase radioimmunoassay to the study of chromosomal components in oviduct is more sensitive, accurate and specific than the conventional serological methods. The monospecific nature of the antibody avoids the possible cross-contamination of other HMG proteins during the assay. We anticipate that this technique will facilitate future studies on chromatin and chromosome structure and function.

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