Changes in quantities of high-mobility-group protein 1 in oviduct cellular fractions after oestrogen stimulation

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Antiserum against chick oviduct high-mobility-group protein 1 (HMG 1) has been induced in the rabbit. With this antiserum, immunobiochemical techniques have been used to probe the quantitative change of HMG 1 in the cellular fractions of chick oviduct before or after oestrogen stimulation. HMG 1 is detectable in the cytosol, microsomal and nuclear fraction of the chick oviduct cell. After administration of oestrogen to young chicks *in vivo* for 5 days, the quantity of HMG 1 is increased 4-fold in the cytosol, 3.5-fold in the microsomal fraction and 1.6-fold in the nuclear fraction. The finding of large amounts of HMG 1 in cytoplasm of oviduct cell is not likely due to its leakage from the nucleus. We anticipate that HMG 1 is synthesized in the cytoplasm and then transported into the nucleus. The synthesis and transportation of HMG proteins is probably regulated by oestrogen.

The HMG proteins are a new group of chromosomal non-histone proteins that were first discovered by Goodwin *et al.* (1973). They can be extracted from chromatin with low-salt solution (0.35 M) or 5% HClO₄ (Goodwin *et al.*, 1975; Rabbani *et al.*, 1978). The proteins bear many resemblances to histones, but are distinct proteins and can be distinguished by a comparison of the amino acid composition. The HMG proteins have an unusual composition in that they contain equal amounts of basic and acidic residues that constitute approximately half of the total amino acid complement (Johns *et al.*, 1975). The HMG proteins are widely distributed in eukaryotic cells, and consist of four major proteins designated as HMG 1, 2, 14 and 17 (Goodwin *et al.*, 1975; Vidali *et al.*, 1977; Watson *et al.*, 1977). A large body of biochemical evidence has been accumulated that correlates these proteins as integral components of genome structure (Alfageme *et al.*, 1976; Watson *et al.*, 1977; Spiker *et al.*, 1978). In addition, the increase in HMG-protein synthesis in co-ordination with cellular proliferation has been demonstrated in rat testis, chicken skeletal muscle and rat liver cell during regeneration (Seyedin & Kistler, 1979; Kuehl, 1979).

In chick oviduct, the cellular growth and proliferation are under the control of oestrogenic hormone. The cytodifferentiation of tubular gland cells is accomplished after 5 days of oestrogen stimulation, and the ovalbumin gene is active for

transcription at this time (Kohler *et al.*, 1969; Oka & Schimke, 1969; Andrews & Teng, 1979). This provides an interesting system to study whether a chromatin-associated non-histone protein is subject to change during oestrogen-mediated cellular differentiation. In a previous publication, we described the isolation and purification of HMG 1 from chick oviduct (Teng *et al.*, 1979). Antiserum against this protein has been induced in the rabbit. By applying this antiserum and the techniques of immunobiochemistry, the subcellular distribution of the HMG 1 proteins and their response to oestrogenic effect have been investigated.

Materials and methods

Fertile White Leghorn chicken eggs, obtained from Rich-Glo Farms, Houston, TX, U.S.A., were placed in a humidified incubator (Petersime model 4) at 38°C. The female chicks were injected 5 days after birth individually with 2.5 mg of oestradiol-17 β in 0.5 ml of propylene glycol daily for 5 days; the control chicks were injected with the vehicle without oestrogen. The chicks were killed by cervical dislocation and the oviducts were removed for nuclear preparation.

HMG 1 and 2 were isolated from nuclei of hen oviduct and purified to homogeneity as described by Teng *et al.* (1979). HMG 17 proteins were isolated by the method of Walker *et al.* (1977). HMG 1 (1 mg) was suspended in 0.5 ml of 10 mM-Tris/HCl, pH 7.4, then mixed with an equal volume of Freund's complete adjuvant and injected intradermally into a

Abbreviations used: HMG, high-mobility-group (protein); IgG, immunoglobulin G.

male New Zealand White rabbit. Two rabbits were individually injected with 500 μ g of HMG 1 in 0.5 ml on days 1, 7 and 15 of immunization. An intramuscular boost, consisting of 250 μ g of HMG 1 in Freund's incomplete adjuvant, was given to each rabbit 1 week later for 3 weeks. The rabbits were bled at weekly intervals starting 1 week after the final boost.

Indirect immunofluorescence of the cultured cells was employed (Miller *et al.*, 1977). The slides containing the fibroblasts from immature chick oviduct were first fixed in 3% (v/v) formaldehyde for 20 min and then in acetone for 7 min. The solutions were precooled at -20°C . The slides were incubated with 50 μ l of anti-(HMG 1) serum (the optimal concentration has been found to be 0.1 mg/ml) at 37°C for 30 min. The cells were then incubated with 50 μ l of anti-(rabbit IgG) goat antiserum with fluorescein label at 37°C for 30 min. Excess serum was removed from the slides and a coverslip was mounted in 99% (v/v) glycerol with Kronig wax. Fluorescence was observed with a Zeiss fluorescence photomicroscope.

The chick oviducts, with or without oestrogen treatment *in vivo*, were homogenized in 5 vol. of 0.32 M-sucrose/3 mM-MgCl₂ with a Polytron P-10 tissue disintegrator (Brinkman, Westbury, NY, U.S.A.) as described by Teng *et al.* (1971). After homogenization, the nuclei were sedimented by centrifugation at 27000 g for 15 min. The nuclear

homogenized suspension was filtered through glass wool to remove fibrous material, and a 4 ml sample was layered over 1.0 ml of an 85% (v/v) glycerol/15% (v/v) α -chlorohydrin mixture chilled to 2°C in a cellulose nitrate tube. Tubes were centrifuged at 110000 g in a Spinco SW50.1 rotor for 30 min at 4°C . The sedimented nuclei were collected for HMG 1 extraction as described above.

The micro-complement-fixation procedures were performed as described by Wasserman & Levine (1961) with minor modification (Teng & Teng, 1980). The protein concentration of HClO₄ extract obtained from different cellular fractions was determined, and 1–500 ng of protein was used for the reaction. The reaction mixture consists of 0.5 ml of antiserum (1:1000, v/v), 0.5 ml of complement (1:150, v/v), various concentrations of antigen in 3 ml of buffer (3 mM-barbital buffer, pH 7.4, containing 0.6 mM-MgCl₂, 0.1 mM-CaCl₂, 200 mM-NaCl and 0.1% bovine serum albumin). After incubation at 2°C for 18 h, sensitized sheep erythrocytes were added and haemolysis was allowed to proceed at 37°C for 90 min. After incubation, unlysed sensitized cells were sedimented by centrifugation at 1000 g for 10 min, and the A_{413} of the haem in the supernatant fluid was determined. The antigen and antibody control did not give evidence of independent pro- or anti-complement activity. The results were expressed as the percentage of complement fixed, calculated as:

$$\text{Percentage of complement fixed} = \frac{\Delta A (A_{\text{control}} - A_{\text{reaction}})}{A_{\text{control}}} \times 100$$

pellet was further purified by suspension in 2.2 M-sucrose and centrifuged at 105000 g for 1 h. The supernatant of the 27000 g-centrifugation step, containing mitochondria and microsomal fraction, was further centrifuged for 15 min at 56000 g to remove mitochondria. The resulting supernatant was centrifuged at 105000 g for 30 min to obtain the microsomal fraction in the pellet and cytosol fraction in the supernatant (Teng *et al.*, 1971). The nuclear, microsomal and cytosol HMG 1 protein was extracted with 5% (w/v) HClO₄ and precipitated with 6 vol. of acetone as described by Rabbani *et al.* (1978) and Bustin & Neihart (1979). The HClO₄-extractable proteins were dissolved in 10 mM-Tris/HCl before immunological tests.

In some experiments, nuclei were prepared from frozen oviduct in non-aqueous media as described by Kirsch *et al.* (1970). The oviduct tissues (1–2 g) were immediately frozen at -80°C and freeze-dried under vacuum for 48 h. The dried tissues were ground with a milling device and suspended in 30 ml of glycerol. The suspension was homogenized with a Polytron P-10 tissue disintegrator with a pulse frequency of 3000 cycles/s for 100 s at 2°C . The

A specific technique for quantification of HMG 1 in the HClO₄-soluble fraction by radioimmunoassay was developed as described by Yuspa *et al.* (1980). Purified hen oviduct HMG 1 proteins were labelled with ¹²⁵I by the chloramine-T method originally developed by Hunter & Greenwood (1962). A portion of Na¹²⁵I (1.0 mCi in 2 μ l) was dispensed into a polystyrene tube containing 10 μ l of 0.25 M-phosphate buffer, pH 7.5. Subsequently, 10 μ l (4 μ g) of HMG 1 solution, 10 μ l (50 μ g) of chloramine-T solution, and 100 μ l (120 μ g) of sodium metabisulphite solution were added. The volume of the solution was made up to 1.0 ml with KI solution (2.0 mg of KI/ml of 0.05 M-phosphate buffer, containing 2% horse serum). The separation of labelled HMG 1 from unchanged iodide was accomplished by chromatography on a column (0.9 cm $\times$ 12 cm) of Sephadex G-25 and eluted with phosphate/serum buffer. ¹²⁵I-labelled HMG proteins were retained for radioimmunoassay. All dilutions for the radioimmunoassay were made in 10 mM-Tris/HCl buffer, pH 7.5, containing 1% bovine serum albumin. In a final volume of 0.5 ml were added: 0.1 ml of rabbit anti-(HMG 1) serum (1:500 initial dilution), 0.1 ml

of ^{125}I -labelled HMG 1 (60–80 $\mu\text{Ci}/\mu\text{g}$, about 60000 c.p.m.) and 0.1 ml of standard HMG 1 from hen oviduct or 20–50 μl of unknown sample (in 10 mM-Tris/HCl buffer) and adjusted to 0.5 ml with the dilution buffer. This mixture was incubated for 2 h at 37°C, followed by 18 h at 4°C. Then 0.1 ml of goat anti-(rabbit IgG) antibody (0.2 mg) was added and the mixture was incubated for 6 h at 4°C. Finally, 1.5 ml of 10 mM-Tris/HCl buffer, pH 7.5, was added to each tube and the immunoprecipitate was isolated by centrifugation at 1200g for 20 min at 4°C. The supernatant was decanted and the radioactivity of the pellets counted in a Beckman Gamma Counter.

Results and discussion

Administration of purified HMG 1 to two rabbits produced serum containing antibody against this protein. The IgG fraction of antisera was tested against the pure HMG 1 by using double-immunodiffusion plates. Only one precipitation line was found (Fig. 1). These antisera were used for the subsequent studies on cellular localization and quantification of HMG 1 protein. The fibroblasts from immature chick oviduct were cultured on glass slides, and HMG 1 distribution in the cell was revealed by immunofluorescence microscopy with anti-(HMG 1) serum. Fig. 2 demonstrates that both

nucleus and cytoplasm are fluorescent-labelled. Bustin & Neihart (1979) also made this observation. They have reported that preferential localization of a protein specifically interacts with anti-(HMG 1) serum in the cytoplasm of various cell types. Recently, they were able to isolate this cytoplasmic protein from hepatoma cells and identify it as an HMG 1 protein that was similar to that obtained from the nucleus (Isackson *et al.*, 1980). On the other hand, in our system, a high intensity of the fluorescence was observed in the nucleus, which supports the other finding, by Rechsteiner & Kuehl (1979). In their micro-injection experiment they were able to demonstrate the transport of HMG protein to the nucleus of HeLa cells in culture. It has been estimated that approx. 70% of the total radioactive HMG proteins are finally localized in the nucleus.

Our observations indicated that, in chick oviduct, HMG 1 is present in the cytoplasmic fractions. Although we are unable to prove the similarity of this protein to that of the nucleus, all the serological studies (immunofluorescence, immunoprecipitation, micro-complement-fixation and radioimmunoassay) have given a positive indication that this is HMG 1 or at least its closely related precursor.

HMG 1 in cytosol, microsomal and nuclear fractions of immature chick oviduct (with or without oestrogen treatment *in vivo*) has been extracted with 5% HClO_4 . This HClO_4 -soluble protein is more abundant in the cytosol and less in the microsomal fraction (Table 1). After oestrogen treatment the total protein in these three fractions increased 4-, 3- and 1.5-fold respectively.

Fig. 1. Immunological reaction between HMG 1 and its antiserum by immunodiffusion: immunodiffusion studies with purified HMG 1 protein

Wells 1+2 and 4+5 contain purified antiserum from two individual rabbits respectively; wells 3 and 6 contain control serum obtained from these two rabbits before they received HMG protein; the centre well contains 25 μl (50 μg) of HMG 1.

Fig. 2. Localization of HMG 1 protein in chick fibroblast by immunofluorescence

The techniques for cell culture and immunofluorescence were described in the Materials and methods section. The fluorescence photomicrograph was taken with Kodak Ektachrome film (ASA 200). The arrows point to nucleus (N) and cytoplasm (C). Magnification $\times 1040$.

Table 1. Amounts of HMG 1 in subcellular fractions

Oviducts of newborn chicks, aged 5 days to 1 week old, with or without receiving oestrogen treatment, were fractionated into three subcellular fractions. Each fraction was extracted with 5% HClO_4 and the soluble fraction was precipitated with acetone. The total protein content in HClO_4 -soluble fractions was determined by Lowry's method (Lowry *et al.*, 1951). The DNA content in the oviduct was determined by the diphenylamine method as modified for this system by Teng & Teng (1978). The content of HMG 1 in HClO_4 -soluble fractions was determined by radioimmunoassay technique described in the Materials and methods section. In a separate experiment, nuclei were isolated with a non-aqueous medium. After obtaining the nuclei, the HClO_4 -soluble fraction was extracted and protein determined as described before. The values presented are means $\pm$ S.D. for three to five determinations.

Cellular fraction	[Protein] ($\mu\text{g}/\text{mg}$ of DNA)						
	Control		HMG 1 (%)	Oestrogen-treated		HMG 1 (%)	stimulation (fold)
	HClO_4 -soluble	HMG 1		HClO_4 -soluble	HMG 1		
Cytosol	265 ± 14.3	1.05 ± 0.13	0.4	1087 ± 56.8	4.20 ± 0.55	0.4	4
Microsomal	26 ± 1.35	0.16 ± 0.02	0.6	78 ± 3.86	0.56 ± 0.07	0.7	3.5
Nuclear	117 ± 5.73	2.11 ± 0.26	1.8	180 ± 9.30	3.36 ± 0.43	1.9	1.58
Nuclear (non-aqueous medium)	121 ± 6.29	2.30 ± 0.32	1.9	183 ± 9.12	3.63 ± 0.44	2.0	1.57

The relative quantity of HMG 1 in the HClO_4 -soluble protein fraction has been probed by an immuno-micro-complement-fixation technique. Fig. 3 indicated that, at a fixed amount of the antigen (200 ng of HClO_4 -soluble protein), the reactivities in cytosol, microsomal and nuclear fractions are 22, 48 and 85% for the control oviduct and 28, 65 and 96% for the oestrogen-treated oviduct respectively. This indicated that, in the nuclei, the HClO_4 -extractable protein has the higher content of HMG 1, which is 3–4-fold and 1.5–2-fold higher than that extracted from the cytosol and microsomal fraction respectively. In order to obtain more specific quantities of HMG 1 in the HClO_4 -soluble fraction, it was further measured by radioimmunoassay. Since polyacrylamide-gel electrophoresis shows that the HClO_4 -soluble fraction consists of four HMG proteins and histone H1 (Fig. 4), whether any cross-reactivity between anti-(HMG 1) antiserum and other HMG proteins (i.e. -2, -14 and -17) or H1 exists during radioimmunoassay was examined. Histone H1, HMG 1, 2 or 17 protein was individually added as non-radioactive competitor to compete with ^{125}I -labelled HMG 1 in the assay system.

Fig. 5 indicates that HMG 1 is one of the most effective competitors among the four proteins tested. HMG 2, HMG 17 and histone H1 slightly compete with radioactive HMG 1 in the assay. In the presence of 1000 ng of the proteins, HMG 1 was able to compete for 97% of the radioactive HMG 1, but only 13, 11 and 2% was competed for by HMG 2, HMG 17 and histone H1 respectively. The results could indicate that there is some contamination of HMG 1 in these preparations; more likely, it is due to the various HMG proteins being immunologically related. A similar observation has been reported in calf thymus by Bustin *et al.* (1978). Therefore the amount of HMG 1 protein detected in this assay is

Fig. 3. Micro-complement-fixation of HMG 1 in cellular fractions of control (open symbols) or oestrogen-induced chick oviduct (closed symbols)

HMG 1 protein or HClO_4 -extractable protein was fixed with rabbit antiserum against hen oviduct HMG 1 protein. The procedure for microcomplement-fixation was performed by using the following antigens: ∇ , HMG 1; $\blacktriangledown$, histone H1; $\circ$, $\bullet$, cytosol protein; $\triangle$, $\blacktriangle$, microsomal protein; $\square$, $\blacksquare$, nuclear HClO_4 -soluble protein (the closed symbols represent the oestrogen-treated chicks); ----, control serum incubated with HMG 1.

slightly affected by the presence of other HMG proteins.

The percentage of HMG 1 in the HClO_4 -soluble fraction remained approximately the same in control and oestrogen-treated chick oviduct, which is 0.4, 0.6 and 1.8 for the cytosol, microsomal and nuclear fractions respectively (Table 1). This result correlated well with our observations presented in Figs. 2

Fig. 4. Polyacrylamide-gel electrophoresis of the HMG proteins extracted with HClO_4

(a) Total HMG proteins extracted from calf thymus nuclei; (b) HClO_4 -soluble protein (from chick oviduct nuclei) precipitated with 6 vol. of acetone. The gel electrophoresis with 10% (w/v) polyacrylamide gel and 2% (w/v) SDS was performed as described by Teng & Teng (1980). H1 is histone H1.

and 3 that the content of HMG 1 protein in nuclei is higher than that in the other two fractions investigated.

The amount of HMG 1 protein in the HClO_4 -soluble fraction was measured with radioimmunoassay and expressed as $\mu\text{g}/\text{mg}$ of DNA. After oestrogen stimulation, the amount of HMG 1 in cytosol, microsomal and nuclear HClO_4 -soluble fractions increased from 1.05 to 4.2, 0.16 to 0.56, and 2.1 to 3.4 respectively, which is a 4-, 3.5- and 1.6-fold increase in these three respective fractions (Table 1). Although the amount of HMG 1 in the cytoplasm is relatively lower than that in the nucleus, it is greatly induced during oestrogenic action. In contrast, a relatively lower induction of HMG 1 protein was found in the nucleus and chromatin. One could argue that the immunodetectable HMG 1 in the cytoplasm is due to the leakage of this protein from the nucleus during the nuclear-isolation process. The diffusion of some nuclear proteins into cytoplasm has been reported

Fig. 5. Standard curve for the radioimmunoassay of HMG 1 protein

The details of the assay techniques are described in the Materials and methods section. ^{125}I -labelled HMG 1 was added to the assay medium containing rabbit anti-(HMG 1) serum at a final dilution of 1:2500. Increasing amounts of HMG 1 (or other proteins) from hen oviduct were added as non-radioactive competitors, and the decrease of radioactivity in the immunoprecipitate was expressed as a percentage of the 'non-competitive' (no HMG 1 protein) control value. Each point represents the mean of six different determinations. ●, HMG 1; ○, HMG 2; △, HMG 17; and □, histone H1.

(Krohne & Franke, 1980). Therefore we have applied a non-aqueous-medium method to obtain the chick oviduct nuclei. There was approx. 6% more HMG 1 obtained from this nuclear preparation than that obtained with an aqueous medium, indicating a leakage of HMG 1 protein. However, this small amount of protein could not contribute to the total content of HMG 1 protein detected in the cytosol and microsomal fractions.

From this observation it is possible that HMG 1 can shuttle between cytoplasm and nucleus in chick oviduct cell. After oestrogen treatment, HMG 1 or its precursor is actively synthesized and stored in the cytoplasm. Most likely, only a proportion of it will be transported into the nucleus. In general, studies on nuclear or chromosomal non-histone proteins in HeLa cells have shown an equilibrium exists between cytoplasmic, nucleoplasmic or chromosomal fractions (Comings & Harris, 1976; Peterson & McConkey, 1976). Furthermore, it is well known that one of the chromosomal components, histone, is synthesized in the cytoplasm and then transported

into the nucleus (Bloch & Brack, 1964; Robbins & Borun, 1967). HMG proteins probably follow the same synthetic pattern. Thus the finding of HMG 1 in the microsomal and cytosol fractions is not surprising.

In eukaryotic cells, the chromosomal non-histone proteins are involved in the regulation of genomic activity (Teng *et al.*, 1971; Kostraba & Wang, 1975). The stimulation of non-histone-protein synthesis by oestrogen treatment has previously been reported in the chromatin of rat uterine tissue and chick oviduct (Teng & Hamilton, 1969; Spelsberg *et al.*, 1973; Teng & Teng, 1980). The stimulation of HMG 1 synthesis in chromatin is a new addition to these related observations. Current observations suggest that HMG proteins are required for constructing an active transcription site in eukaryotic genome (Vidali *et al.*, 1977; Levy *et al.*, 1979; Weisbrod *et al.*, 1980). During the transformation of inactive gene to active gene, there are more newly synthesized HMG protein molecules required to cope with the structural change in the genome. In chick oviduct, after the maximal induction of oestrogen, the epithelia are fully proliferated for multiple gene expression, i.e. ovalbumin, conalbumin, ovalmucoid and lysozyme genes (Palmiter, 1972; McKnight & Palmiter, 1979). More active sites are expected to be opened up for transcription. At present, the manner in which the HMG proteins regulate the genomic activity is still under speculation. In addition, the equilibrium of HMG proteins between cytoplasm and nucleus, and the stability of these endogenously synthesized HMG 1 proteins are still unknown and need to be investigated.

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