Immuno-Biochemical Studies of a Non-Histone Chromosomal Protein in Embryonic and Mature Chick Oviduct

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A non-histone chromosomal 95K protein (of mol.wt. 95000) from hen oviduct was isolated and purified for antibody induction in the rabbit. Immuno-micro-complement-fixation and biochemical techniques were used to probe the presence of 95K protein in the oviduct chromatin of the embryonic and immature chick and the hen. The antiserum against 95K protein did not react with high-mobility-group proteins 1 and 2 obtained from oviduct, brain and liver, nor with histones. After limited digestion of chromatin with nucleases, until 10% of DNA was hydrolysed, a small amount of 95K protein was released. Thus the 95K protein is probably not located in the region of chromatin that is sensitive to nuclease digestion. The amount of 95K protein in immature chick oviduct chromatin is less than that in the mature hen oviduct. However, the amount of 95K protein in the immature chick oviduct was increased after oestrogen administration. The presence of 95K protein in embryonic oviduct was detected in the 10-, 12-, 15- and 18-day chick embryo. The quantity of this protein increased with the age of the embryo and reached its highest value in the chromatin of the hen oviduct.

We have previously observed that in the embryonic chick oviduct (Müllerian duct) a chromosomal non-histone protein (mol.wt. 95000) increases in amount with age (Teng & Teng, 1978a). We subsequently reported the isolation and characterization of this non-histone protein (designated 95K protein) from the mature hen oviduct (Teng *et al.*, 1978). This protein is tissue-specific, and it has a high content of acidic and basic amino acids and low content of aromatic amino acids (Teng *et al.*, 1978). Its characteristics are comparable with those of HMG proteins reported in various tissues of different organisms (Goodwin *et al.*, 1975; Watson *et al.*, 1977; Rabbani *et al.*, 1978; Spiker *et al.*, 1978; Teng *et al.*, 1978, 1979). In hen oviduct the 95K protein is concurrently isolated with the HMG proteins (Teng *et al.*, 1979); therefore we have tentatively classified this protein as one of the HMG proteins.

In the present paper, we report: the induction of an antibody to the 95K protein and its subsequent use to probe the nuclear and genomic localization of the 95K protein; its developmental onset; and its response to oestrogenic-hormone stimulation.

Abbreviations used: HMG protein, high-mobility-group protein; SDS, sodium dodecyl sulphate.

Materials and Methods

Animals and chemicals

Sexually mature White Leghorn hen (50–60 weeks old) oviduct and fertilized White Leghorn chick eggs were obtained from Texas Animal Specialties (Humble, TX, U.S.A.). The chick embryos were incubated at 38°C in a humidified Petersime model 5 incubator at various stages of development and Müllerian ducts were collected as previously described (Teng & Teng, 1978a). All chemicals used in the following experiments were described in previous publications (Teng *et al.*, 1978, 1979).

Isolation and purification of 95K protein

The 95K protein was isolated from the chromatin of hen oviduct nuclei by the method originally described by Goodwin *et al.* (1973) and modified by Teng *et al.* (1978). Chromatin was extracted with 0.35 M-NaCl/0.1 mM-phenylmethanesulphonyl fluoride. The extract was fractionated by 2% (w/v) trichloroacetic acid, and the supernatant was precipitated with 3 vol. of acetone at 0°C. The precipitate, containing 95K protein and H1 histones, was redissolved in borate buffer (7.5 mM-boric acid, 10 mM-2-mercaptoethanol and 0.5 mM-phenyl-

methanesulphonyl fluoride, pH 8.8) and subjected to two steps of CM-cellulose column-chromatographic purification to obtain pure 95K protein (Teng *et al.*, 1978).

Gel electrophoresis of chromatin protein

The 95K protein (or other fraction of protein) was examined by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970) or slab-gel electrophoresis as described by Teng *et al.* (1979).

Immunochemical probe of 95K protein

Antiserum to the 95K protein was produced as follows: 1 mg of pure 95K protein 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 male New Zealand White rabbit. Two rabbits were individually immunized with 500 μ g of 95K protein in 0.5 ml on days 1, 7 and 15 of immunization. An intramuscular boost, consisting of 250 μ g of 95K protein, was given to each rabbit 1 week later. Bleedings were taken 1 week after the booster. The sera from the two individual rabbits were combined and heat-treated at 56°C for 40 min to inactivate the rabbit comple-

ment. The serum was stored at -20°C. The immunoactivity of this antiserum was tested with purified 95K protein or other proteins by the method of quantitative micro-complement fixation (Wasserman & Levine, 1961) in a total volume of 3.5 ml per reaction.

Nuclease digestion of nuclei

Hen oviduct nuclei were suspended in RSB buffer (10 mM-Tris/HCl, pH 7.4, 10 mM-NaCl, 3 mM-MgCl₂, 0.5 mM-phenylmethanesulphonyl fluoride) at a concentration of 2 mg of DNA/ml. The suspension was incubated for various periods at 37°C with pancreatic deoxyribonuclease I (bovine pancreas, activity 1410 units/mg dry wt., at a concentration of 30 μ g/ml) or micrococcal nuclease (*Staphylococcus aureus*, 23882 units/mg of protein, at a concentration of 10 μ g/ml). The unit of nuclease activity was defined by Teng *et al.* (1979). The reaction was terminated when a certain percentage of DNA digestion was reached.

In order to determine the percentage of DNA digestion the following experiments were performed: DNA content was determined by heating an appropriate sample of nuclei (25 μ l/ml) in 1 M-NaCl/1 M-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of nuclear proteins

The proteins were solubilized with 2% (w/v) SDS in 0.0625 M-Tris/HCl, pH 6.8, 0.7 M-2-mercaptoethanol, 5 mM-EDTA and 10% glycerol. Electrophoresis with 10% (w/v) polyacrylamide gel was performed by the method of Laemmli (1970). (a) Gel (1), standard proteins (a, lysozyme; b, ovalbumin; c, bovine serum albumin); gel (2), total 0.35 M-NaCl-extracted proteins (60 μ g); gel (3), 2% (w/v) trichloroacetic acid-insoluble protein (60 μ g) from the 0.35 M-NaCl extract; gel (4), protein precipitated by 3 vol. of acetone (25 μ g) from the 2% trichloroacetic acid-soluble fraction of the NaCl extract; gel (5), protein precipitated by 6 vol. of acetone (25 μ g) from the fraction after precipitation with 3 vol. of acetone from the 2%-trichloroacetic acid-soluble fraction. The protein band in the region marked d is 95K protein. (b) Gel (1), standard proteins (as in a, gel 1); gel (2) 95K protein (10 μ g) after purification by CM-cellulose column chromatography (Teng *et al.*, 1978).

HClO_4 (Axel *et al.*, 1975) at 85°C for 30 min. The sample was cooled for 30 min at 4°C and centrifuged for 30 min at $1500g$ in a Brinkman model 3200 Microfuge (Eppendorf). The supernatant was removed and the A_{260} of the solution measured. A hydrolysed solution of 1 mg of DNA/ml was considered to have an A_{260} of 26 under these conditions (Schaller *et al.*, 1972). An unhydrolysed sample of nuclei was used as a control for background absorption, which represented less than 3% of the total 260nm-absorbing material. Nuclei were preincubated at 37°C for 5 min before the addition of $40\mu\text{g}$ of DNAase I (freshly dissolved)/ml. Digestion was followed by measuring the increase in 260nm-absorbing material soluble in HClO_4 (at 4°C). Appropriate samples ($50\mu\text{l}/\text{ml}$) were removed and mixed with $1\text{M-NaCl}/1\text{M-HClO}_4$. After 30 min at 4°C , the samples were centrifuged as described above and the A_{260} of the supernatant was determined. All samples were corrected for background absorption (3%). Under these conditions, 10% digestion occurred in 7 min at 37°C , and the final extent of digestion was approx. 50% in 60 min. Digestion rates were determined before the experiment. The digestion was stopped by the addition of 100mM-EDTA (pH 7.0) to give a final concentration of 10mM-EDTA . After 3 min at 4°C the nuclei were sedimented at $4000g$ for 10 min with the JS-13 rotor in the J-21E centrifuge, and the supernatant and pellet were immediately used for the extraction of proteins.

Results and Discussion

Immunochemical detection of 95K protein in nuclear fractions

In a previous publication we have described briefly the isolation and characterization of a non-histone 95K protein from the chromatin of mature hen oviduct (Teng *et al.*, 1978). Subsequently we decided to investigate its distribution in the nuclear fraction and its association with the genome. Most of the 95K protein appeared to be in the 2%-trichloroacetic acid-soluble fraction of the 0.35M-NaCl extract of hen oviduct nuclei (Fig. 1a). Precipitation of the trichloroacetic acid-soluble fraction with 3 vol. of acetone selectively extracted the 95K protein from other low-molecular-weight proteins (Figs. 1a, 4, 5). These low-molecular-weight proteins are histones and HMG proteins (Figs. 1a, 5) (Teng *et al.*, 1979).

The crude 95K-protein preparation precipitated by 3 vol. of acetone (Figs. 1a, 4) was further purified by two steps of CM-cellulose column chromatography (Teng *et al.*, 1978). This purified 95K protein appeared to be a single protein when examined by SDS/polyacrylamide-gel electrophoresis (Fig. 1b).

Fig. 2. Immunological reaction between 95K protein and its antiserum by immunodiffusion

Wells (1) and (2) contain antiserum from two individual rabbits; wells (3) and (4) contain control serum from these two rabbits before receiving the 95K protein. (a) Centre well contains $25\mu\text{l}$ of pure 95K protein ($1.0\text{mg}/\text{ml}$). No sample is added in the unlabelled side wells. (b) Centre well contains $25\mu\text{l}$ of crude 0.35M-NaCl extract (15mg of protein/ ml) of hen oviduct chromatin.

Administration of purified 95K protein to two rabbits produced serum containing antibody against this protein (Fig. 2). Antiserum from rabbit 1 gave one precipitation line, and the antiserum from rabbit 2 gave a tiny extra line against the pure 95K-protein preparation (Fig. 2a). The reason for the difference between these two sera is not known. However, immunoelectrophoresis demonstrated that using antiserum from rabbit 2 against pure 95K protein

Fig. 3. Complement fixation by various quantities of nuclear proteins from hen oviduct and other organs in the presence of rabbit antiserum against 95K protein

The immunospecificity of each individual protein was tested by the micro-complement-fixation method of Wasserman & Levine (1961). The antisera were first purified on DEAE-cellulose columns and the antigen-antiserum incubation was carried out at 37°C for 90 min. The antigens were from: ●, 95K protein; ○, protein precipitated by 3 vol. of acetone from salt extract; △, 2% trichloroacetic acid-insoluble fraction (or low-mobility-group proteins fraction); ▲, HMG proteins 1 and 2 from hen oviduct chromatin; □, protein precipitated by 6 vol. of acetone (see Figs. 1a and 5); ----, control serum incubated with 95K protein.

gave one precipitation line (result not shown). In addition, both antisera were tested against the crude 0.35 M-NaCl extract from hen oviduct chromatin, and only one precipitation line was found (Fig. 2b). This indicated that the antisera are specific for 95K protein in the crude 0.35 M-NaCl extract. The antisera were tested against the protein fractions extracted from the nuclei of hen oviduct, brain and liver. Fig. 3 shows that when the antiserum was tested against a purified 95K protein, the value of the complement fixation reached 99% as the amount of antigen reached 60 ng. No such reactivity was observed when control serum was used. A slight reactivity was observed in the low-mobility-group-proteins fractions and in the fraction precipitable by 6 vol. of acetone (Fig. 3). No reactivity was demonstrated with HMG proteins isolated from oviduct, brain and liver, nor with the total histones, ovalbumin or lysozyme (results not shown). These results confirmed our previous finding that the 95K protein is a unique organ-specific protein which is not likely to be the precursor for other HMG proteins (Teng *et al.*, 1978). However, the 95K protein shared certain characteristics with other proteins

HMG-1, -2, -14, and -17 of thymus, liver and oviduct. These characteristics are: co-isolation with these proteins, same ionic strength required for eluting the protein, comparable isoelectric points, and similar amino acid compositions (Teng *et al.*, 1979; Goodwin *et al.*, 1975; Rabbani *et al.*, 1978; Watson *et al.*, 1977).

In order to probe whether the 95K protein is associated with the portion of the DNA that is susceptible to nuclease digestion, we have used pancreatic deoxyribonuclease I or *Micrococcus* nuclease to digest the nuclei until 10% of the DNA was digested. No release of 95K protein was found in the control sample, which had not been treated with either nuclease. A slight but significant amount of 95K protein was released in the presence of nucleases (Figs. 4a and 4b). This probably indicates that 95K protein is mainly located in the region where it is not susceptible to nuclease digestion. On the contrary, proteins HMG-1 and -2 from hen oviduct or other tissues are localized in the region highly susceptible to nuclease digestion (Vidali *et al.*, 1977; Levy & Dixon, 1977; Levy *et al.*, 1977; Teng *et al.*, 1979). It has been suggested that the active or formally active genes are more susceptible to nucleases than are the non-active genes (Weintraub & Groudine, 1976; Garel & Axel, 1976; Garel *et al.*, 1977; Bloom & Anderson, 1978).

Oestrogenic effect on 95K protein

The antiserum (against 95K protein) was used to probe the 95K protein in the oviduct chromatin of newborn chicks (with or without oestradiol stimulation) or mature hens (Fig. 5). This was performed by fixing the antiserum with chromatin or with the 0.35 M-NaCl extract of the chromatin.

At a fixed amount of antigen (10 µg of protein in the form of chromatin or salt extract), the patterns of micro-complement fixation with antiserum were as follows. The control immature-chick oviduct chromatin has a reactivity less than 5%. However, if the salt extract of the chromatin is used, a reactivity of 35% is obtained. The mature hen oviduct chromatin has 56% reactivity with the antiserum. The salt extract of hen oviduct chromatin increases the reactivity to 98%.

If the chromatin was isolated from the oviduct of immature chicks stimulated with oestradiol *in vivo* (1.25 mg of oestradiol-17β daily/chick for 12 days), the reactivity reached 17%. The salt extract of this oestradiol-stimulated chromatin increased the reactivity to 93%, which is close to the value of 98% obtained for hen oviduct. Our results indicated that the amount of non-histone 95K protein in hen oviduct chromatin is higher than that in the immature chick oviduct. However, this chromatin-associated 95K protein in the immature chick oviduct is subject to oestrogen induction. Stimulation of non-

Fig. 4. Separation of nuclear proteins by slab-gel electrophoresis in the presence of SDS

Nuclei were digested with deoxyribonuclease I or micrococcal nuclease as described by Teng *et al.* (1979). The pellet and supernatant fractions from control and digested nuclei were extracted and analysed for the content of 95K protein as described by Teng *et al.* (1979). Protein samples were prepared as described in the legend of Fig. 1 and separated by electrophoresis in 18% polyacrylamide gel containing SDS and stained with Coomassie Blue. (a) Slot nos: (1) and (10), standard protein markers (conalbumin, bovine serum albumin, γ -globulin, ovalbumin, chymotrypsinogen and lysozyme, numbered 1–6 respectively); (2) and (3), control nuclear pellet and supernatant proteins respectively (0.5 min incubation); (4) and (5), control nuclear pellet and supernatant proteins respectively (5 min incubation); (6) and (7), control nuclear pellet and supernatant proteins respectively (7 min incubation); (8) and (9), nuclear pellet and supernatant proteins respectively after 4% DNA digestion (2 min incubation with deoxyribonuclease I). (b) Slot nos: (1) and (2), nuclear pellet and supernatant proteins respectively after 7% DNA digestion (5 min incubation with deoxyribonuclease I); (3) and (4), nuclear pellet and supernatant protein respectively after 10% DNA digestion (7 min incubation with deoxyribonuclease I); (5) and (6), nuclear pellet and supernatant proteins respectively after 4% digestion with micrococcal nuclease (2 min incubation); (7) and (8), nuclear pellet and supernatant proteins respectively after 6% digestion with micrococcal nuclease (5 min incubation); (9) and (10), nuclear pellet and supernatant proteins respectively after 10% digestion with micrococcal nuclease (7 min incubation). H1, HMG-1 etc. represent histone fraction H1, high-mobility-group protein 1 etc.; the identification of these protein bands was described by Teng *et al.* (1979).

Fig. 5. Complement fixation of chromatin-associated 95K protein of control or oestrogen-induced chick oviduct

Chromatin or the 0.35 M-NaCl extract of the chromatin was fixed with rabbit antiserum against hen oviduct 95K protein. The procedure for micro-complement fixation was performed by using the following antigens: $\square$, 0.35 M-NaCl extract of hen oviduct chromatin; $\blacksquare$, hen oviduct chromatin; $\triangle$, 0.35 M-NaCl extract of chromatin from the oviduct of 20-day-old chick receiving oestradiol treatment for 12 days *in vivo*; $\blacktriangle$, oviduct chromatin from 20-day-old chick treated with oestrogen for 12 days; $\circ$, 0.35 M-NaCl extract of oviduct chromatin from control 20-day-old chick; $\bullet$, oviduct chromatin from control 20-day-old chick.

Fig. 6. Complement fixation of 95K protein in the chromatin of developing chick oviduct

Chromatin isolated from oviduct of the chick at various stages of development was extracted with 0.35 M-NaCl. The extract was micro-complement-fixed with the rabbit antiserum against hen oviduct 95K protein. The antigens were the extract of chromatin from: $\circ$, 10-day embryonic-chick Müllerian duct; $\bullet$, 12-day embryonic-chick Müllerian duct; $\triangle$, 15-day embryonic-chick Müllerian duct; $\blacktriangle$, 18-day embryonic-chick Müllerian duct; $\square$, oviduct of 25-day-old immature chick; $\blacksquare$, mature hen oviduct.

histone protein synthesis by oestradiol treatment has previously been reported in the chromatin of rat uterine tissue by Teng & Hamilton (1969, 1970) and Barker (1971), and in chick oviduct by Spelsberg *et al.* (1973). Furthermore, Cohen & Hamilton (1975) have demonstrated that after oestrogen treatment *in vivo* the synthesis of a group of five non-histone proteins with molecular weights ranging from 16000 to 96000 was stimulated. The functional role of 95K protein in the chick oviduct is not yet known. The possibility of this protein serving as a mediator for hormone action should be investigated.

Developmental onset of 95K protein

The oviduct chromatin of embryonic or newborn chicks or mature hens was extracted with 0.35 M-NaCl, and the extracts were made to react with the antiserum (Fig. 6). At a fixed amount of the antigen (20 μ g of salt extract), the 10-day embryonic-chick oviduct chromatin extract shows a significant amount of reactivity, 19%. When the age of the embryo advanced, the reactivity in the chromatin

extract increased to 51, 60 and 62% for the 12-, 15- and 18-day embryo respectively. The reactivity in hen oviduct chromatin was 98%. The increase in this reactivity in the embryonic organ indicated that the amount of 95K protein in a fixed amount of NaCl extract increased during development. This observation paralleled our previous findings that in the developing Müllerian duct the 95K protein progressively increases with age (Teng & Teng, 1978a).

Throughout the four embryonic stages observed, there was a great increase of chromatin 95K protein in the Müllerian duct of 12- and 15-day chick embryos. Within these 3 days of development, there are great increases of some biochemical events, i.e. the cytoplasmic and nuclear oestrogen receptor concentration, oestrogen-binding capacity, template activity for RNA transcription and the capacity for ornithine decarboxylase induction (Teng & Teng, 1975a,b, 1976, 1978a,b; Andrews & Teng, 1979a,b). It is thus possible that this 95K protein may be involved in gene regulation.

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