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Cloning and Characterization of Oviduct Tubular Gland Cell Tissue-Specific Promoters of White Leghorn Chicken

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Abstract: Lactoferrin belongs to the transferrin family, the major function of which is in regulating the iron concentration in the blood stream and gastrointestinal tract. Lactoferrin has a remarkable antibacterial effect and plays an important role in the regulation of immune response. In a previous study, a full-length cDNA of 2133 bp from Macaca cyclopis lactoferrin gene (*mLF*) was cloned and sequenced. An effective promoter that can specifically drive the egg genes is necessary to establish a transgenic chicken line that can overexpress mLF in its eggs in addition to the viral promoters under development. Conalbumin and ovalbumin are the major proteins in egg white. The promoter regions of these two genes were cloned from the chromosomes of white Leghorn chicken in this study. This study uses fluorescence and luciferase assays to test the activities and tissue specificity of these two genes in primary cell cultures of white Leghorn chicken. The results show that the two promoters did not function in chicken muscle cells and fibroblast cells. However, the conalbumin promoter was active in chicken hepatocytes and oviduct tubular gland cells. By contrast, the ovalbumin promoter was active only in chicken primary oviduct tubular gland cells. Both promoters were regulated by a mixture of steroid hormones. The two promoters were also used to drive mLF expression in chicken primary hepatocytes and oviduct tubular gland cells. The results show that the ovalbumin and conalbumin promoters cloned in this study functioned in the chicken primary cell culture level. In the future, promoters will be further studied or reconstructed for gene transfer application in chicken.

Key words: Lactoferrin, ovalbumin, conalbumin, promoter, tissue specificity, Taiwan

INTRODUCTION

Lactoferrin (LF) is an iron-binding protein that is closely related to the plasma iron-transport protein transferrin. Lactoferrin is a single-chain glycoprotein with a molecular weight of approximately 80 kDa. Lactoferrin is folded into two lobes that show sequence homology with each other and each lobe can reversibly bind one ferric ion along with a synergistic anion, usually bicarbonate. In these respects it closely resembles transferrin although, its affinity for iron is relatively higher, allowing iron to be retained at lower pH values. Lactoferrin is also extremely basic with a pI of 8-9, probably caused by a unique basic region in the N-terminal region of the molecule that is not found in transferrin. A wide spectrum of functions is ascribed to lactoferrin. These functions range from a role in the control of iron availability to immune modulation. Lactoferrin is highly conserved among human, mouse, bovine and porcine species. In a previous study, a full-length cDNA of 2133 bp from a Macaca cyclopis Lactoferrin gene (*mLF*) was cloned and sequenced. The nucleotide sequence of mLF shows high identity (93%) to that of the human Lactoferrin gene (*hLF*).

Like tissue culture and mammary gland bioreactors, the avian oviduct can also potentially serve as a bioreactor. The egg white of the hen consists of several layers secreted upon the yolk during its passage through the oviduct. After ovulation or release of the yolk from the ovary, the oocyte passes into the infundibulum of the oviduct, where it is fertilized if sperm are present. It then moves into the magnum of the oviduct which is lined with tubular gland cells. These cells secrete the egg white proteins including ovalbumin, lysozyme, ovomucoid, conalbumin and ovomucin into the lumen of the magnum where they are deposited onto the avian embryo and yolk.

Steroid hormones such as estrogen, progestin, glucocorticoids and androgens regulate the transcription of the two egg white proteins conalbumin (or ovotransferrin) and ovalbumin in chicken oviduct tubular gland cells (Cochet *et al*., 1979; Dierich *et al*.,

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1987; Lee *et al*., 1978; Palmiter, 1972; Perrin *et al*., 1979; Sanders and McKnight, 1985). In the absence of steroid hormones, a basal level of transcription of the conalbumin gene exists whereas transcription of the ovalbumin gene is undetectable (Hager *et al*., 1980; Hynes *et al*., 1979; Mulvihill and Palmiter, 1977; O'Malley *et al*., 1979). In chicken parenchymal liver cells, serum iron levels regulate the transcription of the conalbumin gene (McKnight *et al*., 1980a). This process is substantially less sensitive to steroid hormones than conalbumin transcription in the oviduct (Lee *et al*., 1978; McKnight *et al*., 1980b). In contrast, these cells never express the ovalbumin gene, despite the presence of functional oestrogen receptor molecules which allow estrogen induction of vitellogenin II gene transcription (Deeley *et al*., 1977; Jost *et al*., 1978; Wilks *et al*., 1981, 1982).

Changes in the rates of ovalbumin and conalbumin gene transcription and mRNA levels were monitored during an entire cycle of estrogen withdrawal and restimulation. The mechanisms by which steroid hormones mediate mRNA transcription and stability are generally assumed to involve transcriptional regulation mediated by nuclear steroid receptors that bind to specific sites near the genes they regulate (Palmiter *et al*., 1981). Conalbumin gene transcription is directly proportional to nuclear receptor levels whereas ovalbumin gene transcription is related to receptor levels in a manner that suggests cooperative interactions among receptors.

Ovalbumin gene expression is up-regulated 200 fold after estrogen administration *in vivo*. This is caused by a 20 fold increase in the transcription of the ovalbumin gene (Sanders and McKnight, 1988) coupled with a 10 fold increase in mRNA stability (Arao *et al*., 1996). Estrogen induction of the ovalbumin gene requires two cis-acting regulatory elements in the 5'-flanking region (Sanders and McKnight, 1988), the Steroid Dependent Regulatory Element (SDRE) which spans from -892-793 (Dean *et al*., 1998; Sanders and McKnight, 1988; Schweers *et al*., 1990) and the Negative Regulatory Element (NRE) which spans from 308-88 (Dillner and Sanders, 2002; Haecker *et al*., 1995; Sanders and McKnight, 1988; Sensenbaugh and Sanders, 1999). Furthermore *in vivo* genomic footprinting has identified three protein-protein complexes that bind to the SDRE after estrogen administration. Their complexes are called the chicken inducible regulatory proteins I-III (Chirp-I-III) (Dean *et al*., 1996, 1998; Schweers *et al*., 1990). The correlation between hypersensitivity and transcription was determined for each region in the chick oviduct where expression of the ovalbumin gene can be controlled by steroid hormones. Four DNase I-hypersensitive regions (I-IV) are centered approximately 0.15, 0.80, 3.2 and 6.0 kb upstream from the mRNA cap site in hen oviducts (Kaye *et al*., 1984). Ovalbumin mRNA levels are high in stimulated chicks where hypersensitive regions are present and drop to very low levels in unstimulated chicks where hypersensitivity is absent (Kaye *et al*., 1986).

This study shows that the ovalbumin and conalbumin promoters do not function in chicken fibroblasts, muscle cells and hepatocytes. The exception is the conalbumin promoter which shows basal level activity in primary cultured liver cells. However, both promoters are active in primary cultured chicken oviduct tubular gland cells. This reveals that conalbumin and ovalbumin promoters are not regulated by steroid hormones but show tissue specificity. Based on these characteristics of the conalbumin and ovalbumin promoters, we established a transgenic chicken line that can overexpress mLF protein driving by the conalbumin and ovalbumin promoters. Use of the conalbumin and ovalbumin promoters can avoid the biological dangers of virus promoters.

MATERIALS AND METHODS

Primary cultured cells: Chick embryo liver cells were prepared from 18 days old chicken embryos with minor modifications. After collagenase treatment, the cells were filtered through a nylon sieve (48 μm mesh) before centrifugation. The plated cells were incubated in Petri dishes for several hours in DME medium supplemented with 10% FBS and then Maintained in MEM medium containing insulin (1 mg mL^{-1}) and antibiotics.

Primary cultured oviduct cells were prepared as follows. Oviduct magna were removed from 3 months old chickens. The oviducts were washed 3 times in sterile Phosphate Saline Buffer (PBS), minced with scissors in PBS and trypsinized gently (0.5% trypsin in PBS-EDTA 1mM) for 5 min at 37°C. The supernatant was decanted and made 5% in FBS to inhibit trypsin action. The trypsinization of the pellets was repeated three or four times. All supernatants were pooled and filtered through gauze. The filtered cells were pelleted, resuspended in fresh DME medium supplemented with 10, FBS, 1% L-glutamine, 1 mg L^{-1} insulin and cultivated in Petri dishes at 37°C in 95% air and 5% CO_2.

Plasmids construction: The fluorescence experiment in this study used Enhanced Yellow-green Fluorescent Protein (EYFP) expressed from the pEYFP-Nuc vector as the reporter. pEYFP-cona and pEYFP-oval were constructed by replacing the CMV promoter of the pEYFP-Nuc plasmid with the conalbumin and ovalbumin promoters, respectively.

For luciferase assay, pGL3-Cona and pGL3-Oval were constructed by inserting the conalbumin and ovalbumin promoters into the Kpn I-Bgl II sites and Kpn I-Xho I sites, respectively, upstream of the luciferase gene.

pEGFP-cona-mLF and pEGFP-oval-mLF were used in the Western blotting experiment. To synthesize pEGFP-mLF, mLF was amplified by PCR using the pGEM-T-mLF as a template and primers containing cloning sites (5'-CCGCTCGAGCGGCCGCCACCATGAAGCTTGTCTTC CTCG-3' and 5'-CGGGATCCCGCTTC-TGGAGGAAT-GCACAGG-3') and cloned into the Xho I-BamH I sites of the pEGFP-N1 plasmid. The CMV promoter of the pEGFP-N1 plasmid was then replaced with the conalbumin and ovalbumin promoters to produce pEGFP-cona-mLF and pEGFP-oval-mLF plasmids.

Cell culture and transfection: Primary cultured cells were cultured in DMEM medium supplemented with 10% FBS at 37°C in 95 air and 5% CO_2. When a confluent monolayer formed, the cells were trypsinized, washed and resuspended in fresh DMEM medium. Approximately, 5×10^5 cells were mixed with 15 μg plasmids in a 0.2 cm pulser cuvette. Transfection was performed using the ELECTRO CELL MANPULATOR ECM®2001 (BTX) with a pulse length of 10 (1-99), voltage or 150 V and one pulse. Cells were cultured at 37°C after electroporation.

For the fluorescence experiments, the green-yellow fluorescence was observed by fluorescence microscopy 48 h post-transfection.

Luciferase assay: About 1×10^6 cells were seeded in a 10 cm tissue culture dish and subsequently transfecting with 15 μg of luciferase reporter plasmids and 15 μg of pSV-β-galactosidase control plasmid (Promega) using electroporation. The cells were harvested 48 h after transfection. Transcriptional activity was measured with a luminometer. Conalbumin and ovalbumin promoter plasmids (pGL3-cona, pGL3-oval) were constructed by replacing Simian Virus 40 (SV40) promoters with conalbumin and ovalbumin promoters, respectively. The SV40 promoter (pGL3-control) was used as a positive control. Plasmid pSV-β-Galactosidase directing β-galactosidase expression from the SV40 promoter was used and β-galactosidase assay was performed with the same cell extracts for normalization of transfection efficiency. All of the data shown in this study were obtained from at least three independent experiments.

Western blot and ELISA analysis: The expression of mLF-GFP recombinant protein was detected by Western blot analysis and quantified by ELISA.

For Western blotting, samples prepared from cell lysates were separated by 10% SDS-PAGE. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Millipore) with a semidry horizontal Apparatus (AMMS). The human lactoferrin antibody was used as the primary antibody and Horseradish Peroxidase (HRP) conjugated secondary antibodies were used as the secondary antibody. The molecular mass of each protein was determined using a prestained protein ladder.

For ELISA analysis, the first and second antibodies were rabbit anti-hLF antibody (Sigma) and HRP-conjugated mouse anti-rabbit IgG, respectively. Absorbance was read at 410 nm with a microplate reader. The expression level was calculated according to the standard curve derived from a serial dilution of the yeast-expressed mLF recombinant protein (from previous research). Plates were coated overnight with yeast-expressed mLF recombinant protein diluted with 0.1 mol L^{-1} $NaHCO_3$. Diluted mLF samples were then applied to these wells in duplicates and hLF antibody was detected with HRP-conjugated antirabbit IgG.

Statistical analysis: Student's t test was performed for statistical evaluation of the results. This study presents results as the arithmetic mean with the variance of the mean (mean±SE).

RESULTS AND DISCUSSION

The conalbumin and ovalbumin promoter regions are active in primary cultured chicken oviduct tubular gland cells.

The 5'-flanking regions of conalbumin and ovalbumin genes were cloned from genomic DNA of white Leghorn chicken blood. To check the cloned 5'-flanking regions, the PCR products were sequenced and then aligned with the gene bank sequence (Fig. 1).

To confirm the 5'-flanking regions of conalbumin and ovalbumin genes, pEYFP-cona and pEYFP-oval recombinant plasmids were microinjected into primary cultured chicken oviduct tubular gland cells in the fluorescence experiment and pGL3-cona and pGL3-oval recombinant plasmids were used for the luciferase assay.

Figure 2a shows the different promoter activities in different primary cultured cell types. After transfecting control vectors pEYFP-Nuc, pEYFP-cona and pEYFP-oval recombinant plasmids by electroporation, fluorescence microscopy revealed enhanced yellow-green fluorescent protein. This shows that conalbumin and ovalbumin promoters could drive the enhanced yellow-

(a)

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I    1   agcaagggaa ctcatgtcac aacttgggaa ttttccatt- gaagcccctt gcaaaaacaa 59
II       ---------- ---------- ---------- ---------t ---------- ----------
     60  agagcacctt gcctgctcca gctcctggct gtgaagggtt ttggtgccaa agagtgaaag 119
         ---------- ---------- ---------- ---------- ---------- ----------
     120 gcttcctaaa -atgggctga gccggggaag gggggcaact tgggagctat tgagaaacaa 178
         ---------- a--------- ---------- ---------- ---------- ----------
     179 ggaaggacaa acagcgttag gtcattgctt ctgcaaacac agccagggct gctcctctat 238
         ---------- ---------- ---------- ---------- ---------- ----------
     239 aaaaggggaa gaaagaggct ccgcagccat cacagaccca gaggggacgg tctgtgacca 298
         ---------- ---------- ---------- ---------- ---------- ----------
     299 acaccgctgc tccccctcct caacacccag ctccctgccc caac 342
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(b)

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I    1    ctgcagactg acatgcattt cataggtaga gataacattt actgggaagc acatctatca 60
II        ---------- ---------- ---------- ---------- ---------- ----------
     61   tcacaaaaag caggcaagat tttcagactt tcttagtggc tgaaatagaa gcaaaagacg 120
          ---t------ ---------- ---------- ---------- ---------- ----------
     121  tgattaaaaa caaaatgaaa caaaaaaaat cagttgatac ctgtggtgta gacatccagc 180
          ---------- ---------- ---------- ---------- ---------- ----------
     181  aaaaaaatat tatttgcact accatcttgt cttaagtcct cagacttggc aaggagaatg 240
          ---------- ---------- ---------- ---------- ---------- ----------
     241  tagatttcca cagtatatat gttttcacaa aaggaaggag agaaacaaaa gaaaatggca 300
          ---------- ---------- ---------- ---------- ---------- ----------
     301  ctgactaaac ttcagctagt ggtataggaa agtaattctg cttaacagag attgcagtga 360
          ---------- ---------- ---------- ---------- ---------- ----------
     361  tctctatgta tgtcctgaag aattatgttg tacttttttc ccccattttt aaatcaaaca 420
          ---------- ---------- ---------- ---------- ---------- ----------
     421  gtgctttaca gaggtcagaa tggtttcttt actgtttgtc aattctatta tttcaataca 480
          ---------- ---------- ---------- ---------- ---------- ----------
     481  gaacaatagc ttctataact gaaatatatt tgctattgta tattatgatt gtcctcgaa 540
          ---------- ---------- ---------- ---------- ---------- ----------
     541  ccatgnaaca ctcctccagc tgaatttcac aattcctctg tcatctgcca ggccattaag 600
          -----a---- ---------- ---------- ---------- ---------- ----------
     601  ttattcatgg aagatctttg aggaacactg caagttcata tcataaacac atttgaaatt 660
          ---------- ---------- ---------- ---------- ---------- ----------
     661  gagtattgtt ttgcattgta tggagctatg ttttgctgta tcctcagaat aaaagtttgt 720
          ---------- ---------- ---------- ---------- ---------- ----------
     721  tataaagcat tcacacccat aaaaagatag atttaaatat tccaactata ggaaagaaag 780
          ---------- ---------- ---------- ---------- ---------- ----------
     781  tgtgtctgct cttcactcta gtctcagttg gctccttcac atgcacgctt ctttatttct 840
          ---------- ---------- ---------- ---------- ---------- ----------
     841  cctattttgt caagaaaata ataggtcaag tcttgttctc atttatgtcc tgtctagcgt 900
          ---------- ---------- ---------- ---------- ---------- ----------
     901  ggctcagatg cacattgtac atacaagaag gatcaaatga aacagacttc tggtctgtta 960
          ---------- ---------- ---------- ---------- ---------- ----------
     961  ctacaaccat agtaataagc acactaacta ataattgcta attatgtttt ccatctccaa 1020
          ---------- ---------- ---------- ---------- ---------- ----------
     1021 ggttcccaca tttttctgtt ttcttaaaga tcccattatc tggttgtaac tgaagctcaa 1080
          ---------- ---------- ---------- ---------- ---------- ----------
     1081 tggaacatga gcaatatttc ccagtcttct ctcccatcca acagtcctga tggattagca 1140
          ---------- ---------- ---------- ---------- ---------- ----------
     1141 gaacaggcag aaaacacatt gttacccaga attaaaaact aatatttgct ctccattcaa 1200
          ---------- ---------- ---------- ---------- ---------- ----------
     1201 tccaaaatgg acctattgaa actaaaatct aacccaatcc cattaaatga tttctatggt 1260
          ---------- ---------- ---------- ---------- ---------- ----------
     1261 gtcaaaggtc aaacttctga agggaacctg tgggtgggtc acaattcaga ctatatattc 1320
          ---------- ---------- ---------- ---------- ---------- ----------
     1321 cccagggctc agccagtgtc tgtacataca gctagaaagc tgtattgcct ttagcagtca 1380
          ---------- ---------- ---------- ---------- ---------- ----------
     1381 agctcgaaag 1390
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Fig. 1: a) Alignment of conalbumin and b) ovalbumin. I is obtained from the gene bank. II is the sequence we cloned. Dash means the nucleotide of II is identical to the nucleotide of I

green fluorescent protein expression in primary cultured oviduct tubular gland cells (Fig. 2a-I). In contrast, these two promoters were inactive in primary cultured fibroblast cells and primary cultured muscle cells (Fig. 2a-II, III). It exhibits yellow-green slightly in the field of the primary cultured embryonic liver cell transfected the pEYFP-cona (Fig. 2a-IV) but not pEYFP-oval.

A luciferase assay was used to confirm and quantify the promoter activity. The pGL3-control plasmid was transfected as a positive control and pGL3-basic was used as a negative control. We first lysed the primary cells to release luciferase and then measured its activity. The quantification of conalbumin and ovalbumin promoter activities in different cell types of primary cultured cell showed that the conalbumin promoter has similar activities in both primary cultured oviduct gland cells and liver cells. However, the ovalbumin promoter is active only in primary cultured oviduct gland cells. In the other primary cultured cells including fibroblast cells and muscle cells, neither the conalbumin promoter nor the

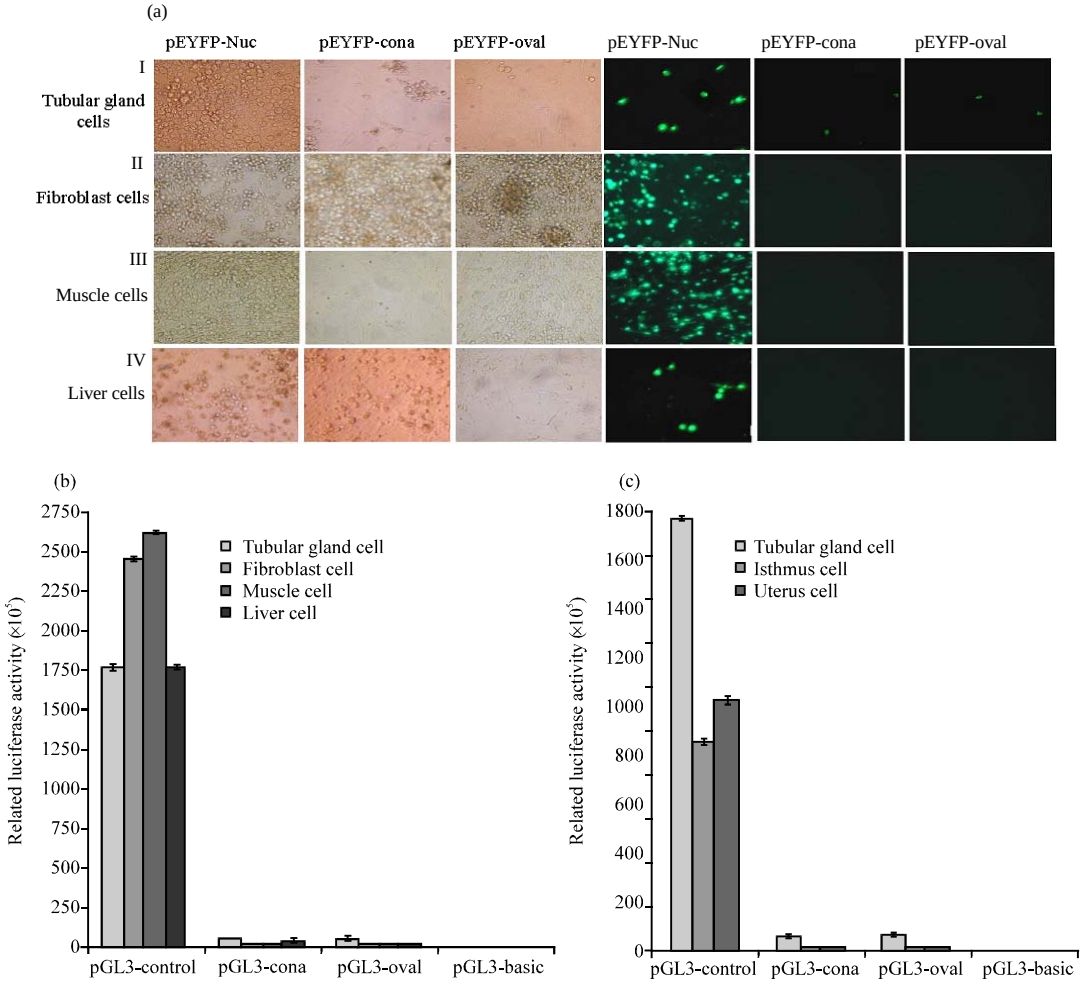

Fig. 2: a) Analysis of conalbumin and ovalbumin promoter activities in different primary cultured cells; b, c) observation of EYFP. Primary cultured cells were injected with the EYFP constructs as described in methods. About 2 days later expression of the reporter was visualized in primary cultured cells using a fluorescent microscope. Expressing embryos were photographed using a digital camera, Luciferase reporter assay for conalbumin and ovalbumin promoter activities. The Luciferase constructs containing conalbumin (pGL3-cona) or ovalbumin (pGL3-oval) promoters were transfected into different cell types of laying hens including; b) tubular gland cell, liver cell, fibroblast cell and muscle cell; c) or different segments of oviduct including tubular gland cell, isthmus cell and uterus cell. The cells were harvested 48 h after transfection for luciferase activity assays. The relative luciferase activities were normalized separately by using β-galactosidase values measured in the same cell extracts as the internal control. Data are represented as mean±SD of triplicate wells from three independent experiments

ovalbumin promoter was active (Fig. 2b). The synthesis of egg white proteins in the magnum including ovalbumin and conalbumin was significantly higher when an ovum was present than in any other segments such as isthmus and uterus (Muramatsu *et al*., 1991, 1994). To test this, we transfected pGL3-cona and pGL3-oval recombinant plasmids into primary cultured isthmus and uterus cells and measured luciferase activity 48 h after electroporation. The results demonstrated that the promoter activities of both conalbumin and ovalbumin promoters are higher in the primary cultured oviduct tubular magnum gland cell than in isthmus or uterus cells (Fig. 2c).

Combined, the 5'-flanking regions of the cloned conalbumin and ovalbumin reacted in a tissue-specific fashion. The activity of the ovalbumin and conalbumin promoter regions is controlled by steroid hormones in primary cultured chicken oviduct tubular gland cells but not in other cell types. The chicken oviduct system has

contributed extensively to the knowledge of the hormonal regulation of gene expression. Estrogen induces several egg white proteins including ovalbumin and conalbumin (Kohler *et al*., 1969; O'Malley and McGuire, 1968; O'Malley *et al*., 1967). Characterization *in vivo* (Oka and Schimke 1969a, b; Palmiter, 1971; Palmiter and Haines, 1973; Palmiter and Wrenn, 1971) and *in vitro* using oviduct explant cultures (Compere *et al*., 1981; Hager *et al*., 1980; McKnight, 1978) has revealed that progestins androgens and glucocorticoids can also regulate the genes for these proteins if the chicks are estrogen treated. To test whether the cloned conalbumin and ovalbumin promoters reflect on steroid hormone treatment, all conalbumin and ovalbumin promoter recombinants were expressed in microinjected primary cultured oviduct tubular gland cells containing 10% FBS, with oestradiol, progesterone and glucocorticoids at concentrations of ~10^{-11} to 10^{-10} M. Unlike primary cultured oviduct tubular gland cells without hormone treatment, Fig. 3a shows higher EYFP expression.

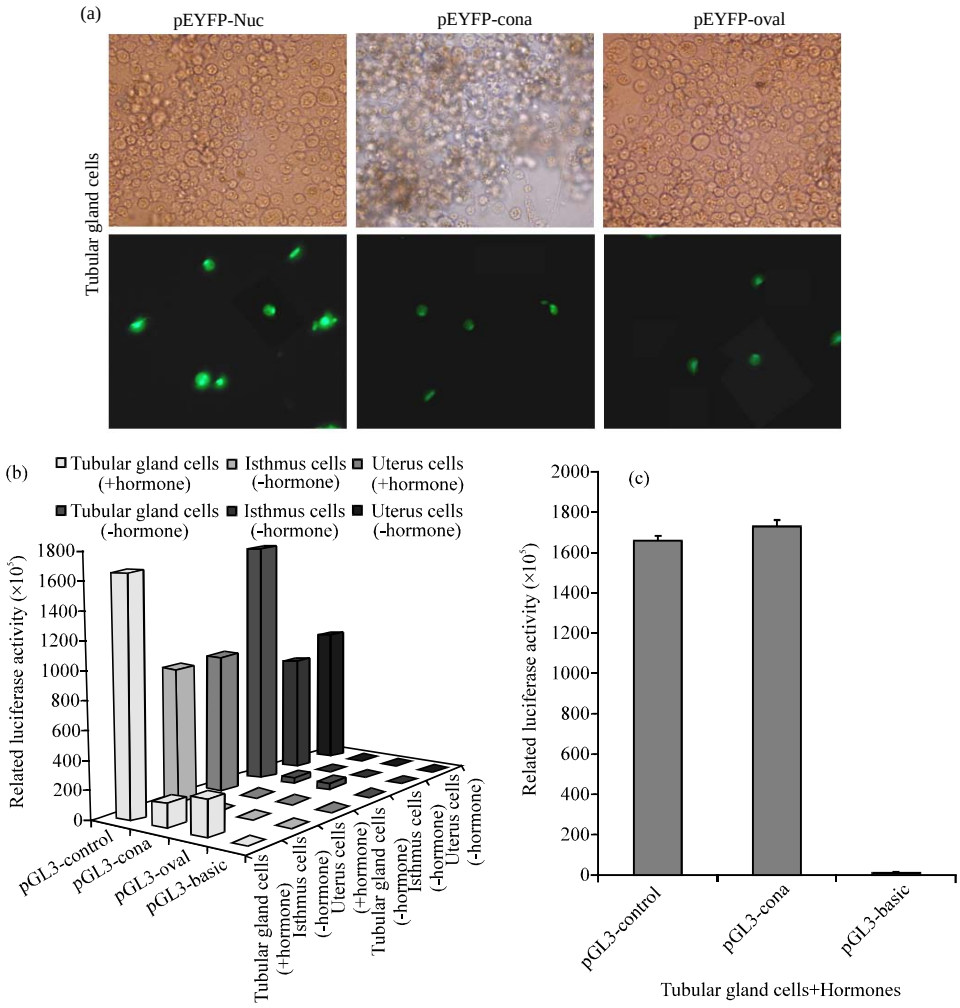

Fig. 3: a) The effect of steroid hormones on conalbumin and ovalbumin promoter activities in different primary cultured cells and b, c) observation of EYFP in primary cultured tubular gland cell treated with steroid hormones (10^{-10} M). Primary cultured tubular gland cell was injected with the EYFP constructs as described in methods. About 2 days later the expression of the reporter was visualized in primary cultured cells using a fluorescent microscope. Expressing embryos were photographed using a digital camera, the results of luciferase assay. The cell-lysated protein samples were freshly prepared from tubular gland cells isthmus cells and uterus cells treated with and without steroid hormones (10^{-10} M). The cells were harvested 48 h after transfection for luciferase activity assays. The relative luciferase activities were normalized separately by using β-galactosidase values measured in the same cell extracts as the internal control. Data are represented as mean±SD of triplicate wells from three independent experiments

Whether treating the hormones affects different segments of the oviduct, we transfected GL3-cona and pGL3-oval into primary cultured tubular gland cells, isthmus cells and uterus cells maintained in DME medium containing 10% FBS with and without oestradiol, progesterone and glucocorticoids at concentrations of ~10^{-11} to 10^{-10} M (Fig. 3b). Luciferase activity was then detected. These results reveal that both conalbumin and ovalbumin promoters are active in primary cultured tubular gland cells and show higher activity with hormone treatment.

In several instances, the activity of the conalbumin promoter was almost as high as positive control which contains SV40 promoter in primary cultured tubular gland cell of laying hens treated with steroid hormones (Fig. 3c).

These results confirm that steroid hormones up-regulate the activity of conalbumin and ovalbumin promoters.

The expression of the mLF is under the control of conalbumin and ovalbumin promoter regions. The previous studies involved cloning the Macaca cyclopis lactoferrin gene (*mLF* gene). In the present study, to express the exogenous protein, mLF, driven by the conalbumin or ovalbumin promoter, we constructed pEGFP-cona-mLF and pEGFP-oval-mLF recombinant plasmids. mLF-EGFP recombinant protein were detected by Western blotting 48 h after microinjection into chicken oviduct tubular gland cells or chicken embryonic liver cells maintained in DME medium containing 10% FBS with oestradiol, progesterone and glucocorticoids at concentrations of 10^{-10} M. The Western blot results were consistent with observations of EYFP expression and analysis of luciferase assay. mLF-GFP recombinant protein was detectable in tubular gland cell lysates transfected with pEGFP-cona-mLF, pEGFP-oval-mLF and the positive control pEGFP-mLF (Fig. 4a). However in primary cultured liver cells, the results only detected conalbumin promoter and CMV promoter-driven mLF-GFP recombinant protein expression despite its weak expression (Fig. 4b).

The concentration of the pEGFP-mLF which served as a positive control containing the CMV promoter increased 10.5 fold compared with the lysate-only negative control whereas the concentration of pEGFP-cona-mLF and pEGFP-oval-mLF increased 3.5 and 5.25 fold, respectively (Fig. 5). Thus, the expression of mLF-GFP recombinant protein was driven by both promoters of conalbumin and ovalbumin genes in primary cultured tubular gland cells.

A highly expressed oviduct promoter has not been developed. This is primarily because an established line of oviduct cells for promoter testing and an efficient

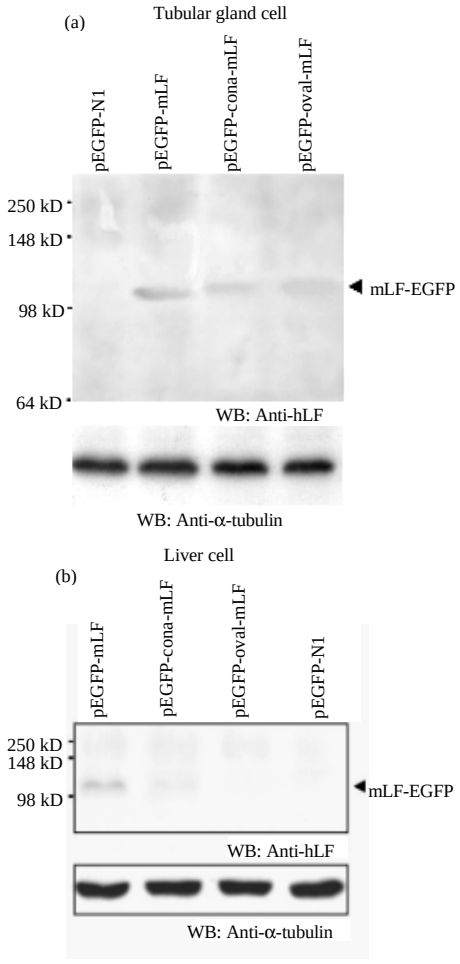

Fig. 4: Western blot analysis of mLF-GFP proteins from primary cultured tubular gland cells and liver cells. The cell-lysated protein samples were freshly prepared from; a) primary cultured tubular gland cells and b) liver cells treated with steroid hormones including β-estradiol, progesterone and corticosterone at the concentration of 10^{-10} M and separated on 10% SDS-PAGE (200 mg proteins/lane) followed by transferring to the nitrocellulose membranes. The transferred membranes were incubated with the anti-hLF antibody. Anti-α-tubulin antibody against tubulin was used for internal control (bottom lanes). Only the vector pEGFP-N1 was used as the negative control as well as the pEGFP-coan-mLF and pEGFP-oval-mLF containing conalbumin and ovalbumin promoters, respectively and exogenous genes and mLF were test samples. The positive control was a CMV-driven construct, pEGFP-mLF. The experiments were repeated twice with similar results

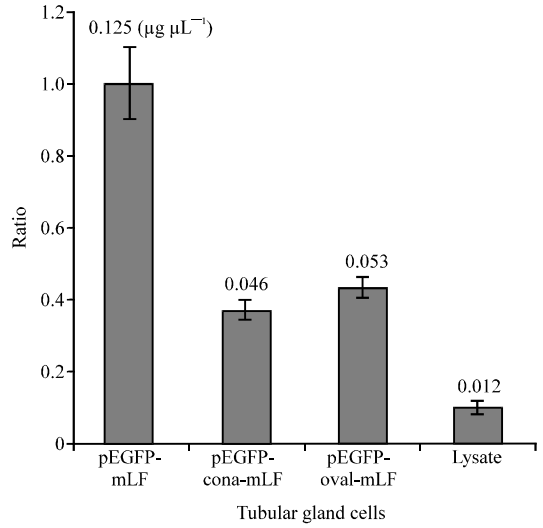

Fig. 5: Ratio of the mLF-GFP recombinant protein expression by ELISA analysis. The three constructs shown in Fig. 4 were analyzed for quantified protein expression. Data are based on three experiments and presented as means±SD

method for producing transgenic chickens has been scant. However, the ovalbumin promoter has been analyzed extensively in explant cultures of immature chick oviducts stimulated to grow and differentiate by steroid hormones (Dean *et al*., 1996; Haecker *et al*., 1995; Sanders and McKnight, 1988; Schweers and Sanders, 1991). The experiments in this study used the primary cultured oviduct tubular gland cells of 3 months old hens and maintained them for eight generations.

To analyze the ovalbumin promoter, four hormonally induced DNase I-hypersensitive sites were generated upstream of the transcription start site (Kaye *et al*., 1984, 1986). These sites most often represent regions in chromatin structurally rearranged by bound transcription factors enabling DNA access for digestion. Proximal sites I (negative regulatory element, NRE) (Haecker *et al*., 1995; Sanders and McKnight, 1988; Sensenbaugh and Sanders, 1999) and II (steroid-dependent response element, SDRE) (Dean *et al*., 1998; Sanders and McKnight, 1988; Schweers *et al*., 1990) are necessary for steroid-mediated expression in chick oviduct explant cultures (Kato *et al*., 1992). The SDRE binds a steroid-inducible protein Chirp-1 (Dean *et al*., 1998, 1996; Schweers *et al*., 1990) and is thought to relieve transcriptional repression at the NRE. Hypersensitive site III contains half-palindromic estrogen response elements that can mediate hormone induction in transfected Hela cells (Kato *et al*., 1992) whereas the function of distal site IV is unknown.

The two proximal sites lie within 850 bp of the transcription start site which is within the ovalbumin promoter region cloned in this study.

The hen has numerous advantages over other systems as a bioreactor. Unlike cows, goats and sheep, chickens have been raised for many generations as specific pathogen-free animals in biosecure facilities. Unlike milk biologics, human and animal vaccines produced in eggs have a long regulatory history. The short generation times and prolific rates of reproduction allow production to be upscaled rapidly. Unlike cell-based production systems which operate most efficiently in batch mode, chickens continuously produce eggs year-round, allowing efficient use of downstream facilities for purification and processing.

CONCLUSION

This study shows that the hen which has high-throughput and low-cost infrastructure is useful for producing exogenous protein such as mLF.

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