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Expression of AGR-2 in Chicken Oviduct during Laying Period

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The chicken oviduct is a dynamic organ that produces secretory proteins such as ovalbumin during the laying period. In this study, we identified oviduct-specific proteins in hens during the egg-laying period by proteomic analysis. Proteins extracted from the magnum of hens of different ages (5, 35, and 65 weeks) were analyzed by two-dimensional gel electrophoresis to compare the intensity of proteins among samples. Approximately 300 spots were detected on each gel. Based on the comparison of image gels, we found that the intensity of eight spots in 35-week magnums was increased at least by 2-fold compared with the others. Five of the eight spots were identified as calumenin, acidic ribosomal phosphoproteins (ARP), prohibitin, heart fatty acid-binding protein, and anterior gradient-2 (AGR-2). In particular, ARP and AGR-2 were highly expressed in 35-week magnums compared with 5- and 65-week magnums. In addition, the level of these proteins was consistent with their RNA levels. Expression of AGR-2 mRNA was detected in the mature magnum, whereas no signal was observed in premature tissue. Among various tissues, expression of AGR-2 mRNA was highest in the magnum, high in the isthmus, and five fold lower in muscle. It was undetectable in the liver and in other tissues (heart and kidney). However, the mRNA levels of other proteins were ubiquitous among tissues. In transcriptional activity of AGR-2, a 3.0 kb fragment of promoter region containing potential estrogen receptor binding sites had enhanced its activity strongly. In conclusion, these results suggest that AGR-2 has functional regulatory roles in the chicken oviduct during the egg-laying period.

Keywords: AGR-2, Chicken, Magnum, Oviduct, Proteome

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Introduction

The avian oviduct is a tubular organ responsible for forming the egg by the secretion of the components surrounding the yolk. It is divisible into five regions: infundibulum, magnum, isthmus, uterus, and vagina. The magnum and isthmus are involved in egg formation (Draper *et al*., 1972; Fernandez *et al*., 2003).

Tissue-specific gene regulation is critically important for determining the proper spatial expression of genes that are responsible for functions specific to one or a limited set of cell types or tissues. Ovalbumin gene expression, for example, is limited to the tubular gland cells of the chicken oviduct in the presence of the steroid hormones estrogen and corticosterone (Bloom and Anderson, 1982; Monroe and Sanders *et al.,* 2000). Due to this highly limited expression, the ovalbumin gene provides an excellent model system to study the mechanisms of tissue-specific gene regulation. However, the precise molecular mechanism regarding oviduct-specific proteins remains unclear.

Proteomics is a novel area of research that involves the global analysis of cellular proteins using diverse technologies such as two-dimensional gel electrophoresis, mass spectrometry, and bioinformatics. With its recent development, two-dimensional gel electrophoresis (2-DE) has been seen as an ideal tool for proteome analyses. Although it has shortcomings (e.g. poor ability to separate hydrophobic proteins and trace quantities), the immobilized pH-gradient strips used in one-dimensional gel electrophoresis provide a basis for reproducible separation according to proteins' isoelectric points. Proteome comparison between tissues in different situations could be analyzed. Recently, research concerned with diseases, especially cancer and obesity, has been undertaken using proteomics in humans and mice (Jones *et al.,* 2002; Park *et al*., 2005; Wang *et al*., 2006). However, there are limited studies of the expression of specific proteins in the chicken oviduct.

The purpose of this study was to determine oviduct-specific proteins during the laying period using proteomic analysis. Protein extracts of oviductal tissues from immature hens

(approximately 5 weeks old) and laying hens (approximately 35 and 65 weeks old) were analyzed by 2-DE.

Materials and Methods

Animals. Eggs and oviducts were obtained from the ISA Brown laying hens breed with artificial light provided for 16 h a day and food and water ad libitum. Oviducts were surgically removed from euthanized hens at 5, 35, 55, 65, and 83 week. Various tissues (magnum, isthmus, liver, kidney, heart, and skeletal muscle) were collected (0.5-1 g) and stored at liquid nitrogen until required.

Protein extraction and two-dimensional polyacrylamide gel electrophoresis. Extracts (20% w/v) were prepared by homogenizing in the buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT). The supernatant recovered after centrifugation at 12,000 × *g* was stored −80°C until the 2-DE analysis was performed.

Protein extracts were applied to IPG gel strips pH 3-10L (Amersham Biosciences Inc.). Isoelectrofocusing was conducted using pH 3-10 Pharmalytes. Upon completion of the first dimension, strips were incubated with gentle shaking in an equilibration buffer (50 mM Tris-HCI, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% SDS and trace amount of bromopheol blue) containing 1% DTT and 2.5% iodoacetamide. For second dimension, strips were transferred to 12.5% polyacrylamide gels containing SDS. Gels were run for 12 h using the Ettan DALT system (Amersham Biosciences Inc.) with 2 W/gel at 20°C. After fixation, gels were stained with silver staining kit (Amersham Biosciemces Inc.).

Image acquisition and data analysis. Gels were scanned using an ImageScanner flatbed scanner. Computer-assisted image analysis was performed using ImageMaster 2-D Elite software package (Amersham Biosciemces Inc.). The relative spot volume was directly related to protein concentration. Spot intensities were normalized using ImageMaster software to analyze quantitative changes among samples. Comparative analyses were conducted by matching the gels.

ESI-TOF MS/MS. Proteins were subjected to in-gel trypsin digestion and tandem electrospray mass spectra were recorded using a Q-TOF mass spectrometer. In brief, samples were dissolved with 100 µl of destain solution (30 mM potassium ferricyanide, 100 mM sodium thiosulfate) with shaking for 5 min. After removal of the solution, gel spots were reacted on 200 mM ammonium bicarbonate for 20 min and were dehydrated with 100 µl of acetonitrile. The dried gel pieces were rehydrated with 20 µl of 50 mM ammonium bicarbonate containing 0.2 µg modified trypsin (Promega) for 45 min on ice. After removal of solution, 30 µl of 50 mM ammonium bicarbonate was added. The digestion was performed overnight at 37°C. The peptide solution was desalted using C18 nano column. MS/MS of peptides produced by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (Micromass). The MS/MS data were processed using a Mass Lynx Windows NT PC system. The MS/MS spectra recorded on tryptic peptides derived from spot were searched against protein sequences from NCBInr databases using the MASCOT search program.

RNA extraction and reverse transcription-polymerase chain reaction. Total RNA was isolated from 300 mg of various tissue using the TRizol Reagent (Invitrogen). RNA was eluted with diethyl pyrocarbonate-treated distilled water. Reverse transcription was performed with 1 µg of RNA, using random primers and 200 units of M-MLA reverse-trancriptase (Promega) at 42°C for 60 min. Polymerase chain reactions were performed in 20 µl using 10 ng of cDNA, standard PCR buffer, 1.5 mM $MgCl_2$, 250 µM each dNTP, 100 nmol each primer and 0.5 U AmpliTaq DNA polymerase (Applied Biosystems). The following cycling protocol was used: at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec and a final extension step at 72°C for 10 min. The PCR product was sequenced to confirm its identity. The primer sequences used in this study are listed in Table 1. PCR products was electrophoresed in a 0.7% agarose gel and stained with ethidium bromide for visualization under ultraviolet light.

Real-time quantitative PCR analysis. For the relative quantification of the mRNA levels, cDNA product was used as template in PCR reactions in the presence of SYBR Green I PCR master mix (Bio-Rad Co). The PCR reactions and their analysis were done in an iCycler machine using the iCycler iQ Optical System software (Bio-Rad Co.). The ratio change in target gene relative to the GAPDH control gene was determined by the 2^{-DDCt} method (Livak and Schmittgen, 2001). The primers were designed by the Primer 3 Output program and are listed in Table 1. All experiments were done in triplicate.

Cell culture and promoter assay. HeLa cells were maintained with DMEM supplemented with 10 % fetal bovine serum and 1 mg/ml penicillin-streptomycin. Semiconfluent cells were transiently transfected using ExGen 500 (Fermentas) according to the manufacturer's guidelines. Briefly, 10^5 cells were plated on 3.5-cm tissue culture dishes in DMEM containing 10% FBS and after 24 h cells were transfected with the different constructions of the AGR-2 promoter. The 0.8 kb, 1.5 kb and 3 kb fragments were subcloned in the luciferase reporter vector pGL3 (Promega). Typically, cells received 2 µg luciferase reporter plasmid and 2 µg of an internal control plasmid, pCMV-beta-gal, which contains the gene for beta-galactosidase enzyme. After 6 h of exposure to the ExGen 500/ DNA mixture, the cells were incubated in complete medium for another 18 h. Cells were then washed, incubated in hormone-striped medium containing 17β-estradiol (10 nM), and harvested after 24 h for determination of beta-galactosidase and luciferase activity. Quantification of luciferase activity was performed with TD20/20 luminometer. Each transient transfection experiment was repeated at least three times in triplicate.

Results and Discussion

Proteomic analysis of chicken oviducts. To isolate specific proteins expressed in the mature magnum of chickens, 2-DE was conducted with samples taken from 5-, 35-, and 65-week-old hens. As shown in Fig. 1, the gel derived from 35-week magnums showed abundant ovalbumin compared with 5- and

Table 1. Primer sequences for PCR amplification of the specific genes

Target gene	Primer sequences	Size
Calumenin	*(F) 5'-CAC CCC GAG GAG TAC GAC TA-3' *(R) 5'-TAT TTG GCC ACG ATC TCC TC- 3' **(F) 5'-GAG TTC ACC GCC TTC CTG-3' **(R) 5'-CTC CGT CTT CAC CCA TTC G- 3'	341 bp 177 bp
ARP	*(F) 5'-TCA TGA AAA TCA TCC AAC TG-3' *(R) 5'-GAT GTT CAG CAT GTT CAG CA-3' **(F) 5'-GAG ACC GAC TAC ACC TTC C-3' **(R) 5'-CTC CTC CGA TTC CTC CTT C-3'	500 bp 156 bp
Prohibitin	*(F) 5'-CTC GCC CAC GTA ACA TAC CT-3' *(R) 5'-GAA TTC CTT GCC AAA GGT CA-3' **(F) 5'-GAG CGA GCC AGG TTC ATT G-3' **(R) 5'-AAG TAG GTG ATG TTG CGA GAC-3'	326 bp 200 bp
HFBP	*(F) 5'-ATG GTG GAA GCG TTC GTG GG-3' *(R) 5'-CTA TGA TGC CTT CTC AGA GG-3' **(F) 5'-AGC ACC TTC AAG AAC ACA GAG-3' **(R) 5'-AGT CAG AAT CAA CTT CCC ATC C-3'	400 bp 186 bp
AGR2	*(F) 5'-GCC AAG CAC TCA AGA AGG TC-3' *(R) 5'-TGC TGC CCT GTA CAG AAG TG-3' **(F) 5'-GAG CAG ACA TTA CTG GAA GAT AC-3' **(R) 5'-GTG GAT GAG TGG AGA AGA GG -3'	438 bp 176 bp
GAPDH	(F) 5'-ATG GTG AAA GTC GGA GTC AA-3' (R) 5'-AGT GTC CGT GTG TAG AAT CA-3'	120 bp
28 S	(F) 5'-TTC ACG CCC TCT TGA ACT CT-3' (R) 5'-GCC CAA GTC CTT CTG ATC G-3'	228 bp

*Primer for reverse-transcription -PCR
** Primer for quantitative real-time PCR

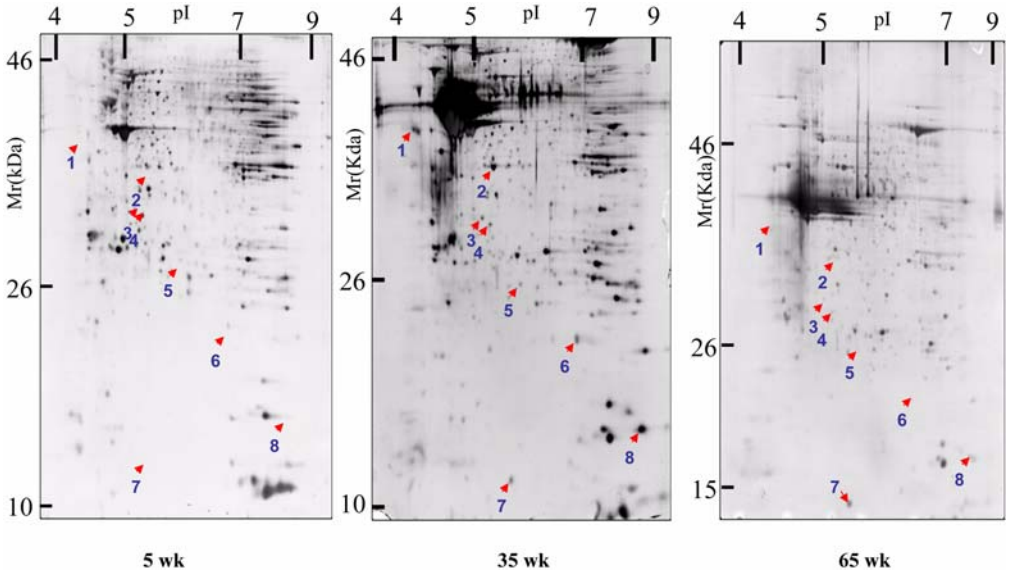

Fig. 1. 2-DE gel map of chicken oviduct. Proteomic analysis was conducted with the magnum of hens of different ages (5, 35 and 65 weeks). Protein spots expressed in the magnum of 35-week hens were compared with those at 5 and 65 weeks. Arrows indicate differentially expressed spots.

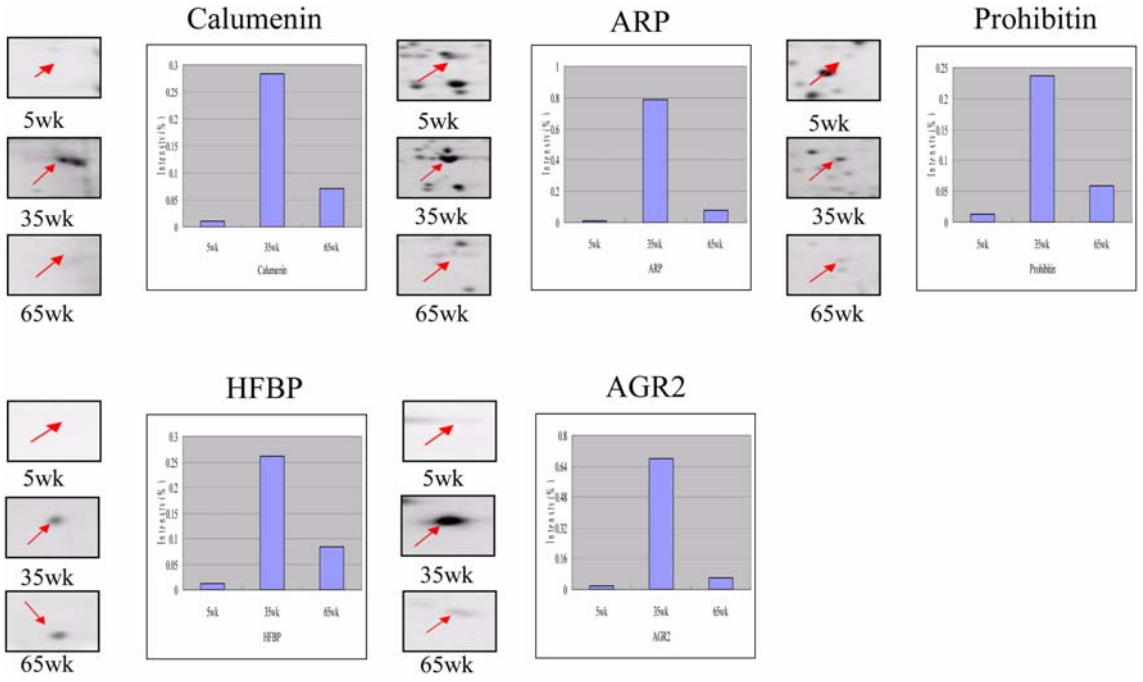

Fig. 2. Magnified comparison maps of the different regions in 2-DE protein patterns between the 35-week magnum and others (5- and 65-week tissues).

65-week tissues, though the same amounts of protein extracts were loaded, suggesting that our samples were derived from hens of different reproductive age.

Overall, 300 spots were detected each gel. To detect proteins specifically expressed in the 35-week magnum, we compared gels between the 35-week magnum and the others (5- and 65-week). The intensity of 20 spots was variable among samples. The signal of eight spots among them was at least 2-fold higher in the 35-week magnum than in 65-week tissues (Fig. 2). As shown in Table 2, these significant spots were identified as acidic ribosomal phosphoprotein (ARP), calumenin, prohibitin, heart fatty acid-binding protein (HFBP), and anterior gradient-2 (AGR-2). Three of the eight spots were unknown proteins. Calumenin is a multiple EF-hand Ca^{2+}-binding protein located in the endo/sarcoplasmic reticulum of mammalian tissues (Yabe *et al*., 1997) . ARP is widely used as an estradiol-independent housekeeping gene. GnRH-I or GnRH-II inhibits the expression of mRNA encoding the ARPs (Chen *et al*., 2002). Prohibitin is highly conserved in eukaryotic cells and is an inhibitor of cell proliferation (Mishra *et al*., 2006). Fatty acid-binding proteins are members of a superfamily of lipid-binding proteins. AGR-2 is a secreted protein encoded by the cement gland-specific genes XAG-1 and XAG-2, expressed in the anterior region of the dorsal ectoderm of *Xenopus* (Aberger *et al*., 1998).

The mRNA level of selected proteins. To gain further insight into the possible role of these proteins in the chicken oviduct, variation in magnum message expression during the egg-laying period was assessed by quantifying the levels of calumenin, prohibin, HFBP, ARP, and AGR-2 mRNA in magnum tissues collected at 5, 35, 55, and 83 weeks. We

Table 2. Identification of protein spots derived from 35 week-magnum

Spot	Protein description	Accession No.	Score	Sequence coverage (%)	pI	Mr
1	Calumenin	AAB 97725	88	30	4.47	37,164
2	Acidic ribosomal phosphoprotein (ARP)	NP_990318	335	23	5.72	34,435
3	Unknown	-	-	-	-	-
4	Prohibitin	AAH54971	69	8	5.28	29,859
5	Unknown	-	-	-	-	-
6	Unknown	-	-	-	-	-
7	Heart fatty acid-binding protein (HFBP)	NP_001026060	64	19	5.92	14,807
8	Anterior gradient-2 (AGR-2)	XP_418698	35	6	8.94	19,943

Table 3. Fold change in gene expression by quantitative real time-PCR

	Control	35-week	55-week	83 week
Calumenin	1	0.5	0.7	1.4
ARP	1	2.76	3.5	10.0
Prohibitin	1	0.1	0.3	0.1
HFBP	1	0.4	9.8	1.3
AGR2	1	97.3	887.3	137.6

*Relative expression was compared to control (5-week oviduct), which were assigned a valve of 1. Each PCR was normalized with a GAPDH internal control as described under Materials and Methods.

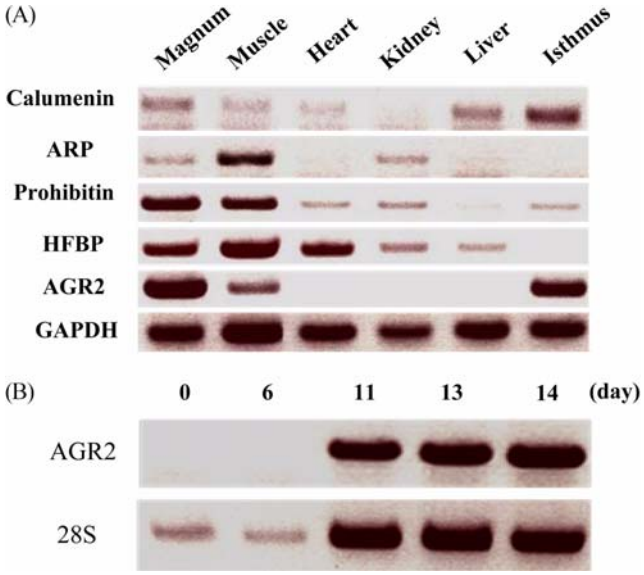

Fig. 3. Expression of mRNA in various tissues of laying hens. (A) RNA was isolated from various tissues such as the oviduct (magnum and isthmus), liver, muscle, heart, and kidney. Following reverse transcription, PCR was performed using designed primers. GAPDH was used as a loading control. (B) Using RT-PCR, the expression of AGR-2 mRNA was determined in eggs during the development period. 28S was used as a control.

could not study the levels of these proteins because antibodies are not yet available. RNA levels of the five proteins were analyzed using real time quantitative-PCR. As shown in Table 3, the expression of calumenin, prohibitin, and HFBP was slightly decreased, whereas ARP and AGR-2 were expressed strongly throughout the active egg-laying period. In particular, the level of AGR-2 mRNA was dramatically increased in mature oviduct. At 55-week hen, its signal was enhanced by 880 fold compared to that of premature tissue. These results suggest that AGR-2 may involve the differentiation process in oviduct epithelial cells during the egg-laying period.

Tissue-specific expression of selected genes. To determine the tissue-specific expression of the five selected genes, we

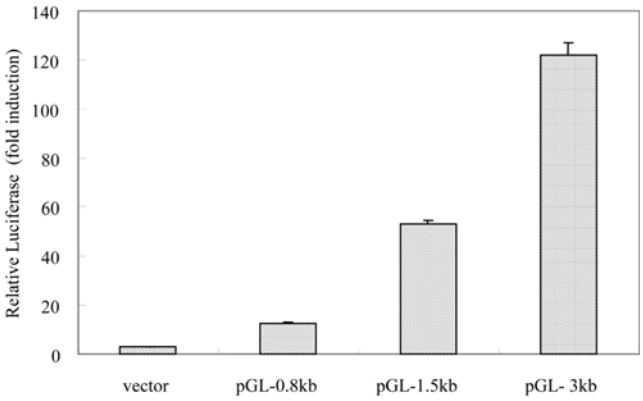

Fig. 4. Activation of AGR-2 promoter by estrogen. The different constructs were transiently transfected into HeLa cells in the presence of 10 nM estradiol, and luciferase assays were performed 24 h after transfections. Data are expressed relative to the values obtained with the vector, and luciferase activity were determined in triplicate in at least three independent experiments.

performed reverse transcriptase -PCR analysis with various tissues such as kidney, muscle, heart, liver, and isthmus of 35-week hens. As shown in Fig. 3A, calumenin was expressed ubiquitously in all tissues. The intensity of ARP was enhanced in skeletal muscle, but was weak in the magnum. Prohibitin was expressed ubiquitously except in liver, and was highly expressed in the magnum. HFBP showed a similar pattern to prohibitin, except for the heart. Interestingly, expression of AGR-2 was limited to the magnum and isthmus portions of the oviduct. The other tissues tested (liver, kidney, and heart) were negative for AGR-2, except muscle, which showed weak expression. This pattern of AGR-2 expression in the oviduct was confirmed quantitatively by proteomic analysis (Fig. 2). In addition, expression of AGR-2 in eggs was increased during development (Fig. 3B). AGR-2 was first detected at embryonic day 7. This is consistent with the important role of *Xenopus* AGR-2, XAG-2, in the development of *Xenopus* embryos (Aberger *et al*., 1998). XAG-2 is implicated in the formation of the cement gland and induction of forebrain fate (Aberger *et al*., 1998).

Promoter activity of AGR-2. To determine the biological activity of AGR-2 promoter, several fragments of the AGR-2 promoter were cloned upstream of the luciferase reporter gene in the pGL1 vector (constructs pGL0.8 kb, pGL1.5 kb, and pGL3 kb) and tested for their expressions in HeLa cells exposed to estrogen. Basal activities are shown in Fig. 4. A 1.5-kb fragment of promoter region of AGR-2 gene (construct pGL1.5 kb) had modest activity, whereas the 3-kb insertion from transcription start of fragment resulted in 100-fold enhancement of the activity compared to that of the pGL-0.8kb construct. These data suggest the presence of strong positive *cis*-elements along the AGR-2 gene promoter. Computer analysis of this 3 kb-flanking sequence revealed a consensus sequence for four potential estrogen hormone

response element at nucleotides 1866 to 1848, 1511 to 1493, 1326 to 1308, and 969 to 951. Based on our results, a 3-kb fragment of AGR-2 promoter contains these 4 potential estrogen receptor binding sites, so this fragment had strong transcriptional activity compared to that of 1.5 kb- and 0.8 kb-fragments of AGR-2 gene promoter. The human homologs, hAG-2 and hAG-3, are secreted proteins that show hormone-dependent expression. They have been shown to be associated with estrogen-positive breast tumors (Thompson and Weigel, 1998; Liu *et al.,* 2005). In laying hens, the uterus and ovary are estrogen-dependent organs and egg laying is affected by the secretion of estrogen (Fernandez *et al*., 2003), so that the increase in AGR-2 may be associated with egg laying. Eggs are formed sequentially as they traverse the oviduct of the hen. The egg white constituents are secreted by the magnum, and the shell membranes assemble on the surface of the egg white from precursors secreted in the white isthmus (Draper *et al*., 1972). Therefore, These results indicate that AGR-2 may be a strong candidate as an oviduct-specific protein involved in egg formation and epithelial cell differentiation during the egg-laying period.

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