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Cloning of Ovocalyxin-36, a Novel Chicken Eggshell Protein Related to Lipopolysaccharide-binding Proteins, Bactericidal Permeability-increasing Proteins, and Plunc Family Proteins*

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The avian eggshell is a composite biomaterial composed of noncalcifying eggshell membranes and the overlying calcified shell matrix. The shell is deposited in a uterine fluid where the concentration of different protein species varies at different stages of its formation. The role of avian eggshell proteins during shell formation remains poorly understood, and we have sought to identify and characterize the individual components in order to gain insight into their function during elaboration of the eggshell. In this study, we have used direct sequencing, immunochemistry, expression screening, and EST data base mining to clone and characterize a 1995-bp full-length cDNA sequence corresponding to a novel chicken eggshell protein that we have named Ovocalyxin-36 (OCX-36). Ovocalyxin-36 protein was only detected in the regions of the oviduct where eggshell formation takes place; uterine OCX-36 message was strongly up-regulated during eggshell calcification. OCX-36 localized to the calcified eggshell predominantly in the inner part of the shell, and to the shell membranes. BlastN data base searching indicates that there is no mammalian version of OCX-36; however, the protein sequence is 20–25% homologous to proteins associated with the innate immune response as follows: lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and Plunc family proteins. Moreover, the genomic organization of these proteins and OCX-36 appears to be highly conserved. These observations suggest that OCX-36 is a novel and specific chicken eggshell protein related to the superfamily of lipopolysaccharide-binding proteins/bactericidal permeability-increasing proteins and Plunc proteins. OCX-36 may therefore participate in natural defense mechanisms that keep the egg free of pathogens.

The avian eggshell is a calcitic bioceramic, which possesses unique mechanical properties deriving from its complex a porous polycrystalline structure. It rests upon, and is continuous with, an underlying noncalcified meshwork of fibers. The eggshell membranes, the calcified (mammillary, cone, and palisade) layers and cuticle of the eggshell, are sequentially laid down upon the egg white in the distal part of the oviduct (isthmus and uterus). The eggshell mineral is associated with an organic matrix composed of proteins, glycoproteins, and proteoglycans, which are thought to influence the fabric of this biomaterial (1). Matrix protein components have been identified in decalcified shell and in the uterine fluid bathing the egg during its formation, and this contains the organic and mineral precursors of the eggshell (2). Previously identified matrix protein components can be divided into three characteristic groups as follows: (i) "egg white" proteins, which are also present in the eggshell, and these include ovalbumin (3), lysozyme (4), and ovotransferrin (5); (ii) ubiquitous proteins that are found in many tissues, and this group includes osteopontin, a phosphorylated glycoprotein present in bone and other hard tissues (6–8), and clusterin, a widely distributed secretory glycoprotein that is also found in chicken egg white (9); and (iii) matrix proteins unique to the shell calcification process that are secreted by cells in specific regions of the oviduct where eggshell mineralization is initiated and continues to completion (red isthmus and uterus). OC-17 (Ovocleidin-17) was the first eggshell protein purified from the shell (10). It is a C-type, lectin-like phosphoprotein of 17 kDa (11) that occurs in glycosylated (23 kDa) and nonglycosylated forms in the shell matrix (12). OC-116 (Ovocleidin-116) was the first eggshell matrix protein to be cloned (13). OC-116 is the protein core of a 120/200-kDa eggshell dermatan sulfate proteoglycan termed ovoglycan (13–15), which is found throughout the compact calcified eggshell (13). Recently, we described OCX-32 (Ovocalyxin-32), a 32-kDa uterine-specific protein that is concentrated in the outer calcified region and cuticle of the calcified shell (16–18).

The natural defenses of the egg have two components as follows: one is the shell that acts as a physical barrier, and the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ968387.

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other is a chemical system composed of endogenous antibacterial proteins that have been mainly identified in egg white (19). It has been shown that changes in eggshell properties are directly related to increasing risk of egg contamination and risk of food-borne outbreaks for consumers (20). In addition, we have shown that proteins with well known antimicrobial properties are specifically associated with the shell. Lysozyme is abundant in the limiting membrane that circumscribes the egg white and forms the innermost layer of the shell membranes. It is also present in the shell membranes and in the matrix and cuticle of the calcified shell (4). Ovotransferrin is localized in the calcified mammillae and particularly in the eggshell membranes, where it functions as a bacteriostatic filter to reinforce inhibition of *Salmonella* growth in egg white (5).

In this study, we report the cloning, cDNA and protein sequence, genomic organization and regulation of expression of Ovocalyxin-36 (OCX-36), a novel eggshell-specific protein that is homologous to members of mammalian antibacterial protein families. This protein is the first example of an eggshell-specific protein that may participate in the natural chemical defense of the egg against bacteria.

EXPERIMENTAL PROCEDURES

Collection of Uterine Fluid—Brown egg-laying hens (ISA brown strain) were caged individually and subjected to a cycle of 14 h of light followed by 10 h of darkness. They were fed a layer mash as recommended by the Institut National de la Recherche Agronomique (21). Each cage was equipped with a device for automatic recording of the time of oviposition (egg laying). Uterine fluid was collected as described previously (2). Egg expulsion was induced by intravenous injection of 50 μg of prostaglandin 2-α/hen at 6–9, 13–20, and 22–23 h after previous oviposition (the ovulation of the following egg coincides with this event, $\pm$0.5 h). These time intervals correspond to the initiation, rapid growth, and final phase of shell mineralization, respectively. Uterine fluid was collected immediately after egg expulsion into a plastic tube placed at the entrance of the everted vagina. Aliquots of uterine fluid were immediately diluted 1:1 with 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.125% bromphenol blue for electrophoresis.

Tissue Collection—Tissues (eight samples for each organ and each stage) of the oviduct (magnum, white and red isthmus, and uterus) and other organs (liver, kidney, duodenum, and muscle) were harvested from birds when an egg was in the magnum (3–4-h post-oviposition, phase of albumen secretion onto the yolk) or during the rapid growth phase of calcification (16–18 h post-oviposition). Additionally, uterine tissues of birds at various physiological phases related to sexual maturity and egg formation were collected from the following: (i) 10 immature birds (15 weeks old) with a developing oviduct before the initiation of egg production; (ii) 10 adult hens in which 50 μg of prostaglandin 2-α was injected during 3 consecutive days to expel the egg before mineralization had begun; and (iii) 10 additional birds in which the uterus was collected during eggshell calcification.

Extraction and Solubilization of Matrix Proteins—Eggshell matrix proteins were extracted as described (5) with slight modifications. The shells were rinsed with running tap water and then washed in 154 mM NaCl. Four methods were used for the preparation of shell to ensure the removal of shell membranes from the inner part of the calcified shell. (i) Membranes were mechanically removed using forceps. (ii) Each egg was filled with 130 mM EDTA, pH 8.5, for 30 min, followed by the mechanical removal of the membranes using forceps. The egg was then filled again with the same solution for another 30 min. (iii) Eggs were filled with 130 mM EDTA, pH 8.5, for 30 min, and the membranes were mechanically removed. The eggs were filled again with 1 N HCl for 5 min. (iv) Eggs were filled with 130 mM EDTA, pH 8.5, for 30 min, and the membranes were mechanically removed. The eggs were filled again with 0.1 N HCl for 20 min. Following these treatments to remove all associated shell membranes, shells were then ground to a fine powder. Powdered shells were then decalcified overnight at 4 °C in 20% acetic acid. The suspension was dialyzed (cutoff 3500 Da) against demineralized water and lyophilized. This powder was extracted overnight at 4 °C with 4 M guanidine hydrochloride in 50 mM sodium acetate, pH 4.8, containing protease inhibitors (5 mM benzamidine-HCl, 100 mM amino-*n*-caproic acid, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). After centrifugation the supernatant was dialyzed against water (cutoff of 3500 Da) and then concentrated. The final preparations of soluble matrix proteins under these conditions are referred to as eggshell protein extracts.

Antibody Preparation—Two preparative SDS-polyacrylamide gradient gels (3 mm width) were loaded with uterine fluid samples harvested at the rapid growth phase of calcification (8 mg of protein). After electrophoresis the gels were stained with Coomassie Blue and then thoroughly rinsed with demineralized water. The 32–36-kDa band was excised and ground into a fine powder with a Spex freezer mill. The powder was suspended in 154 mM NaCl, mixed 1:1 with Freund's complete adjuvant, and injected into two adult female New Zealand rabbits. The animals were boosted seven times, at 3-week intervals, with the same antigen preparation in Freund's incomplete adjuvant to obtain a satisfactory titer.

Additionally, an antiserum (Ab[2] Ocx36-(51–65)) was prepared against a 15-amino acid synthetic peptide corresponding to a sequence near the N terminus of the OCX-36 protein (residues 51–65, KHLQGMALPNIMSDR). The peptide was coupled to keyhole limpet hemocyanin and used for immunization of two adult female New Zealand rabbits. For the first injection (day 0), the antigen (200 μg) was mixed with Freund's complete adjuvant. Subsequent boosting with antigen emulsified in Freund's incomplete adjuvant was performed at days 14, 28, 49, and 70. At day 77, animals were sacrificed and the sera collected.

SDS-PAGE and Electroblotting—The protein concentration of each sample was determined by the Bradford procedure (22) using ovalbumin as the standard. SDS-PAGE and electro-

[2] The abbreviations used are: Ab, antibody; LBP, lipopolysaccharide-binding protein; BPI, bactericidal permeability-increasing protein; RT, reverse transcription; nt, nucleotides; PBS, phosphate-buffered saline; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LC-MS/MS, liquid chromatography/tandem mass spectrometry; INRA, Institut National de la Recherche Agronomique; EST, expressed sequenced tag.

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blotting were performed on a 4–20% gradient gel or 12% isocratic gels as already described (2, 16). Uterine fluid samples and eggshell extracts were prepared in SDS-PAGE buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.125% bromphenol blue) and boiled for 5 min prior to loading onto the gels. Proteins were stained with Coomassie Blue or electroblotted (0.8 mA/cm^2) onto the following: (i) either polyvinylidene fluoride membrane (Hyperbond, Beckman Instruments Inc., Fullerton, CA) in 10 mM CAPS buffer, pH 11, 10% methanol for microsequencing, (ii) or nitrocellulose membrane (Bio-Rad) in 25 mM Tris, 192 mM glycine, 10% methanol for Western blot analysis.

Western Blotting—Nitrocellulose membranes were washed in phosphate-buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) and blocked for 1 h in Odyssey blocking buffer (Odyssey, LI-COR Biosciences Inc., Lincoln, NE). The membranes were then incubated for 60 min with Ab Ocx36-(51–65) (1:3,000 in Odyssey blocking buffer). After four washes (5 min each) in PBS/Tween (PBS, 0.1% Tween 20), the membranes were incubated for 1 h with 1:5,000 Alexa Fluor 680 goat anti-rabbit IgG (Molecular Probes) in Odyssey blocking buffer. The membranes were then washed four times in PBS/Tween and then twice in PBS. The membranes were scanned using an Infrared Imaging System (Odyssey, LI-COR Biosciences Inc., Lincoln, NE) in the 700 nm channel. Prestained molecular weight standards are visible on the blot in this channel.

Microsequencing—N-terminal and internal microsequencing of the 32–36-kDa protein collected at the rapid growth phase was performed. Samples were separated by SDS-PAGE and electrotransfered onto polyvinylidene fluoride membranes. The membranes were stained with 0.1% Coomassie Brilliant Blue, 40% methanol, and 1% acetic acid for a few minutes and destained in 50% methanol until the bands became clearly visible. The 32–36-kDa Coomassie Blue-stained band was excised, directly loaded into a protein sequencer, and subjected to N-terminal sequencing by Edman degradation in an LF 3000 protein sequencer (Beckman Instruments). Alternatively, the 32–36-kDa band was subjected to trypsin digestion for internal microsequencing by standard protocols. The resulting fragments were separated by reverse phase high pressure liquid chromatography and then injected into the protein sequencer.

Mass Spectrometry—The uterine fluid collected at the rapid growth phase was separated by SDS-PAGE under reducing conditions. The Coomassie Blue-stained band corresponding to the 32–36-kDa band was cut with a scalpel into small blocks. The blocks were rinsed and then reduced and alkylated with dithiothreitol and iodoacetamide. They were incubated for 2 h at 37 °C in a microtube with 12.5 ng/μl trypsin (sequencing grade; Roche Applied Science) in 25 mM NH_4HCO_3 as described previously (23). The tryptic fragments were extracted, dried, reconstituted with 0.1% formic acid, and sonicated for 10 min.

Tryptic peptides were analyzed by MALDI-TOF mass spectrometry (MALDI L/R P/N; Waters). The α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml dissolved in 50% ethanol, 50% acetonitrile) and the sample (1:1, v/v) were loaded on the target using the dried droplet method. The analyses were performed in positive ion reflector mode, with an accelerating voltage of 15,000 V. For subsequent data processing, the MassLynx 4.0 software (Waters) was used. Spectra obtained were calibrated externally using the $[M + H]^+$ ions from bovine serum albumin digest. A mass deviation of 0.1 Da was allowed for data interpretation.

TABLE 1
List of primers used for RT-PCR

Primer label	Primer sequence	Position relative to	
		cDNA sequence	Coding sequence
36-H1	AGTCTGAACGTCCGCCTG	1237–1254	1111–1128
36-H2	TAGCAGCAGCATCCCATG	1795–1777	1667–1649
36-F1	TTGGAATGGTCGTCTTCTGTGG	146–167	20–41
36-B1	CGGTCTGAATGATGGCATCG	265–246	139–120
36-F2	GGCATTCTGTCTGAGGTGATCG	466–487	340–361
36-B2	GCAGTCATCAACAGTGACGTGG	573–552	447–426
36-F3	ATTGGGATGCAGTTGGTCGTCACC	409–432	283–306
36-B3	GAAGGTGTATGGCTGGATATGGAG	990–967	864–841

Peptides were sequenced by nano-LC-MS/MS (CapLC coupled to a Q-TOF-Global equipped with a nano-ESI source; Waters). Only doubly and triply charged ions were allowed to be selected as precursors over a mass range of 400–1300 *m*/*z*. The collision energy was selected depending on the precursor ion mass and charge. The mass spectrometer was calibrated using the fragmentation spectrum of Glu-fibrinopeptide (Sigma), 500 fmol/μl in 50:50 water/acetonitrile, 0.1% formic acid. The peptides were loaded on a precolumn (monolithic trap column, 200 μm × 5 mm; Dionex) and desalted with 0.1% formic acid for 5 min at 25 μl/min, followed by separation on a C_{18} column (Atlantis™ dC18; 3 μm, 75 μm × 150 mm; Nano Ease™, Waters), eluted with a 5–60% linear gradient (buffer A: water/acetonitrile 98:2 (v/v) containing 0.1% formic acid; buffer B: water/acetonitrile 20:80 (v/v) containing 0.1% formic acid) over 60 min at a flow rate of 180 nl/min. The peptide masses and sequences obtained were matched automatically to proteins in a nonredundant data base (NCBI) using the Mascot program.

cDNA Expression Screening—The cDNA library was prepared from uterine tissue harvested during the active phase of eggshell calcification as described previously (13). Protein synthesis was induced with 10 mM isopropyl-1-thio-β-D-galactopyranoside. The phage library was screened using antiserum raised to the 32–36-kDa uterine fluid protein (1:1000) and anti-rabbit IgG alkaline phosphatase conjugate (1:3000) as secondary antibody and detected with nitro blue tetrazolium and 5-bromo 4-chloro-3-indolyl phosphate. Phage with positive inserts were purified to homogeneity by three rounds of screening and rescued into Bluescript plasmid using helper phage. Plasmid DNA was isolated by alkaline lysis miniprep, and the cDNA inserts were sequenced by automated protocols at the sequencing service of INRA-Centre de Tours, Nouzilly, France.

Hybridization Screening—A clone with a novel insert of 1019 bases in length was obtained by expression screening (see above). PCR primers were designed using this sequence (36-H1/36-H2; Table 1) and used for RT-PCR to investigate the expression of this message in different segments of the oviduct and in liver. PCR product was only detected in the uterine por-

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tion of the oviduct. The 525-bp PCR product was labeled (digoxigenin, Roche Applied Science) and used for hybridization screening of the same library to obtain longer inserts. In this manner, six independent clones were isolated and sequenced by automated protocols at the BRI sequencing service of the Faculty of Medicine, University of Ottawa, Canada. Two of the clones contained potentially full-length inserts of 1710 bp.

Reverse Transcriptase-PCR and mRNA Levels by Real Time PCR—Total RNA was extracted from frozen tissue samples using a commercial kit (RNeasy mini kit, Qiagen, Courtaboeuf, France) and simultaneously treated with DNase (RNase-free DNase set, Qiagen). RNA concentrations were measured by absorption at 260 nm, and the quality of RNA was electrophoretically evaluated on a 1% agarose gel. Samples of total RNA (2 μg) were reverse-transcribed using RNase H^- Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen) and random hexamers (Amersham Biosciences). Classical PCR was performed to amplify nt 146–265 and nt 409–990 of the OCX-36 cDNA, using the 36-F1 forward/36-B1 reverse primers and 36-F3 forward/36-B3 reverse primers (Table 1) for 30 cycles at 60 and 65 °C respectively.

Alternatively, OCX-36 cDNA was amplified in real time using the qPCR Master mix plus for Sybr Green I (Eurogentec, Seraing, Belgium) with the ABI PRISM 7000 apparatus (Applied Biosystems). To account for variations in mRNA extraction and reverse transcription reaction between samples, OCX-36 mRNA levels were corrected relative to ribosomal 18 S rRNA levels. The latter were measured using a TaqMan universal PCR master mix and predeveloped TaqMan assay reagents for human 18 S rRNA (Applied Biosystems, Courtaboeuf, France) as validated previously (9).

Four specific primers were chosen from the OCX-36 cDNA sequence (Table 1). The combination of 36-F1/36-B1 primers and of 36-F2/36-B2 primers allowed the amplification of 121- and 109-bp fragments, respectively (Table 1). The cycling conditions consisted of a uracil-*N*-glycosylase preincubation step at 50 °C for 2 min, followed by a denaturation step at 95 °C for 10 min, and 40 cycles of amplification (denaturation for 15 s at 95 °C, annealing and elongation for 1 min at 60 °C). A melting curve program was carried out from 60 to 95 °C in 20 min for each individual sample amplified with Sybr Green. Samples showed a single product with a specific melting temperature of 84 °C for the combination of 36-F1/36-B1 and of 78 °C for the combination of 36-F2/36-B2. Each sample was diluted, and then amplified in triplicate in the same run. Each run included triplicates of no template controls and triplicates of control cDNA corresponding to a pool of uterine cDNA from laying hens sampled during eggshell formation. The control cDNA was diluted from 1:50 to 1:156,250, and relative arbitrary quantities were defined. The threshold cycle (C_T), defined as the cycle at which fluorescence rises above a defined base line, was determined for each sample and control cDNA. A calibration curve was calculated using the C_T values of the control cDNA samples, and relative amount of unknown samples were deduced from this curve. The PCR efficiencies for 36-F1/36-B1, 36-F2/36-B2, and 18 S rRNA were 95, 102, and 98%, respectively.

The ratio value was calculated for each sample as OCX-36/18 S rRNA. The log of the ratio was used for statistical analysis using StatView software (SAS Institute Inc. version 5). A one-way analysis of variance was performed to detect significant differences in OCX-36 expression in organs at various physiological phases.

Radiation Hybrid Mapping—Radiation hybrid mapping was performed on the ChickRH6 panel (24). The left primer (gctgaagctttttggaatgg) and right primer (ctgggtgggtgagagcag) were designed using the Primer3 software, available on line. PCR was performed in 15 μl, containing 25 ng of hybrid DNA, 2 mM $MgCl_2$, 0.3 unit of *Taq* DNA polymerase (Invitrogen), 1× buffer (Invitrogen), 200 μM of each dNTP, 0.2 μM of each primer, and 1× loading buffer (350 mM sucrose, 0.2 mM cresol). After denaturation for 10 min at 94 °C, 35 PCR cycles were performed (30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C), followed by a final elongation step of 10 min at 72 °C. PCR products were analyzed on a 1% agarose gel and visualized using ethidium bromide staining. Chicken DNA was used as positive control, and hamster DNA and TE (Tris-EDTA) buffer served as negative controls.

Immunolocalization of OCX-36 in Eggshell and Uterus—Thin sections of oviduct tissues and decalcified eggshell were prepared and immunostained as described previously (3, 10). In brief, tissues from two hens were removed 2 h after oviposition, fixed in ice-cold 4% paraformaldehyde in PBS for 24 h, and then equilibrated in sucrose before dehydration and embedding in paraffin wax. Pieces of shell were fixed and decalcified at room temperature in a 1:1 mixture of 150 mM Na-EDTA, pH 7.7, and 4% paraformaldehyde in phosphate-buffered saline. When samples were sufficiently decalcified (3–4 days on average), they were transferred to PBS to rinse out the decalcifying reagent. To offer support, the shell pieces were pre-embedded flat in heated 2% agar, 10% formalin solution (45 °C). The agar block was solidified at room temperature and then processed as usual in an automatic tissue processor (Triangular Biomedical Systems). Paraffin blocks were prepared with a tissue embedding center (Leica EG 1160) by orienting the agar block to show the cross-section of the egg shell. Sections (4 μm) were dewaxed in xylene and rehydrated through an ethanol series into TBS (100 mM Tris-HCl, pH 7.7, 150 mM NaCl), and then incubated with antiserum raised to the OCX-36 synthetic peptide (Ab OCX-36-(51–65)) at various dilutions in TBS containing 0.3% Triton X-100 for 3 h at room temperature. In some cases, as controls, the primary antiserum was omitted. Sections were washed (two times for 10 min in TBS, 0.3% Triton X-100) and then incubated for 1 h at room temperature with secondary antibody (1:400, sheep anti-rabbit-CY3 conjugate; Sigma). After washing, the immunoreactivity was visualized by fluorescence microscopy.

Additionally, samples were prepared for colloidal gold immunocytochemistry. Samples were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, washed in cacodylate buffer alone, dehydrated in ethanol, and embedded in LR White acrylic resin as described previously (25). Survey sections of embedded tissue were viewed by light microscopy, and selected regions were trimmed for thin

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sectioning and transmission electron microscopy. Grid-mounted tissue sections 80 nm in thickness were processed for immunocytochemistry by incubation with primary antibody, and immunolabeling patterns were detected by a second incubation using protein A-colloidal-gold complex (Dr. G. Posthuma, University of Utrecht, The Netherlands). Incubated grids were conventionally stained with uranyl acetate and lead citrate and examined in a JEOL 2000FX transmission electron microscope operated at 80 kV.

Scanning Electron Microscopy of Eggshell-Membrane Samples—To evaluate the extent of membrane removal from eggshells exposed to various digestion/decalcification treatments, eggshell fragments were dried in air and mounted with conductive carbon paste onto metallic SE stubs to provide an *en face* view from the inner side of the membranes/shell (*i.e.* from the egg white side). Samples were then sputter-coated with a 20–25-nm thick Au-Pd thin film, and morphological imaging was performed using a Hitachi S-4700 field-emission gun scanning electron microscope (FE-SEM) operating at an accelerating voltage of 5 kV.

RESULTS

cDNA Cloning and Genomic Sequence of Ovocalyxin-36—Uterine fluid bathes the egg during its formation and contains eggshell matrix components as soluble precursors (2). Its protein composition varies considerably during each of the three stages of the calcification process when analyzed by SDS-PAGE (Fig. 1*a*, *lanes 2–4*). Particularly evident during the active growth phase is a 32–36-kDa protein (Fig. 1*a*, *lane 3*). Following SDS-PAGE and blotting onto polyvinylidene fluoride membranes, this band was subjected to N-terminal amino acid sequencing to yield the sequence VLSGLSCAIS-PRAMQQV. Furthermore, tryptic digestion of the protein band allowed three additional fragments to be sequenced: fragment 1, AMQQVLSDAIIQTG(G)L; fragment 2, VEIYLPR; and fragment 3, VSLFL*X*S*X*DIG. Fragment 1 was aligned with the N-terminal amino acid sequence to yield the sequence of the first 28 amino acids of the 32–36-kDa uterine fluid protein (VLSGLSCAISPRAMQQVLSDAIIQTG(G)L). Preparative SDS-PAGE allowed the large scale separation of the 32–36-kDa uterine fluid protein. The band was excised from the gel and injected into two rabbits to produce polyclonal antibodies that were used to screen an expression cDNA library prepared with mRNA purified from hen uterine tissue harvested during the active calcification phase. Eight positive clones were thus isolated and sequenced. Of these, four corresponded to Ovocleidin-116, the protein core of a major proteoglycan of eggshell matrix (13), and three to chickens clusterin that we have reported as a 35–40-kDa uterine fluid protein (9). Finally, one clone contained a novel 1019-bp cDNA sequence (Fig. 2).

PCR primers were designed from the novel sequence, and the amplicon was labeled for hybridization screening of the same uterine library to obtain longer inserts. Novel independent clones were isolated and assembled to obtain a composite 1710-bp cDNA sequence (Fig. 2). A BlastN search using the composite sequence identified six ESTs overlapping it. Clones gcal0003c.m.17 (EMBL accession numbers BX273494 and BX273493) and gcag0011c.m.09 (EMBL accession numbers

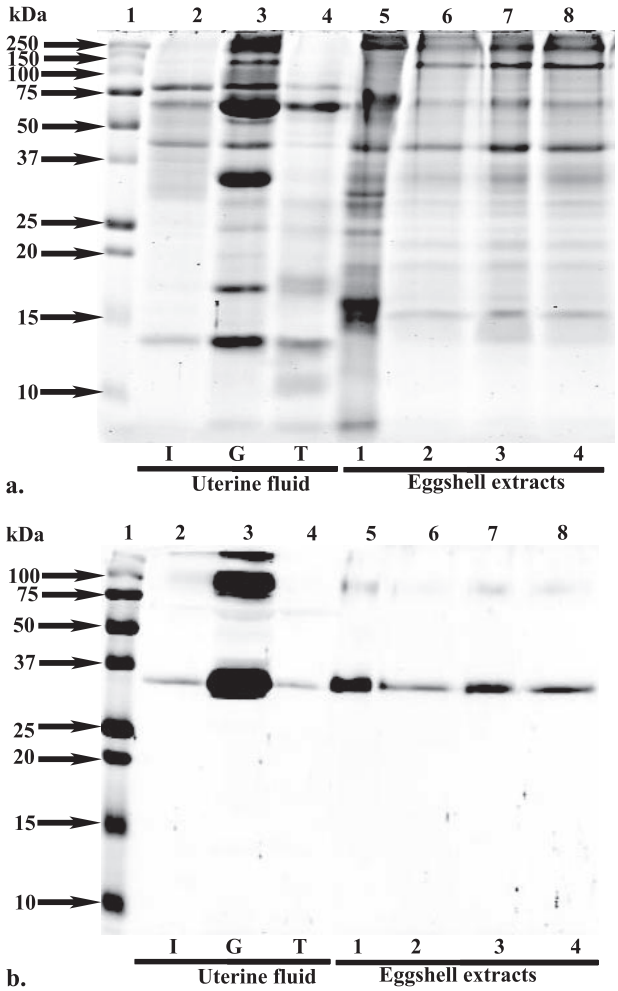


FIGURE 1. **SDS-PAGE and Western blot analysis for OCX-36 in uterine fluid at various stages of shell calcification and in eggshell extracts.** Lanes were loaded as follows: *lane 1*, standard, prestained molecular size markers (size on *left*); *lanes 2–4*, uterine fluid at different stages of shell formation (*I*, initial stage, 11 μg; *G*, active growth phase, 7 μg; *T*, terminal phase, 5 μg); *lanes 5–8*, eggshell protein extracts (10 μg) in which eggshell membranes were removed as described under "Experimental Procedures": *1*, mechanical removal (*lane 5*); *2*, EDTA treatment (*lane 6*); *3* and *4*, combined EDTA and HCl treatments (*lanes 7* and *8*). *a*, gel stained with Coomassie Blue. *b*, Western blotting after electrotransfer to nitrocellulose membrane.

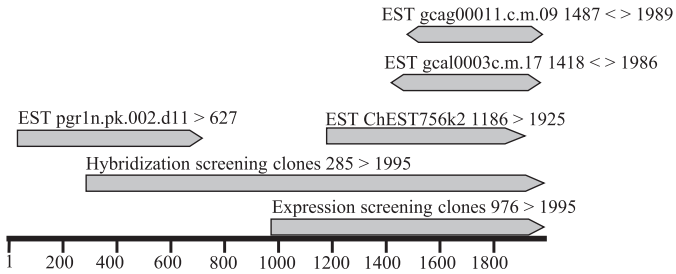


FIGURE 2. **Schematic representation of the various clones used for the determination of the OCX-36 full-length cDNA.** Positions of clones according to the consensus sequence are indicated by *numbers*. Direction of sequence is indicated by 〈 and 〉 (5′ to 3′ and 3′ to 5′ of the consensus sequence, respectively) and by the *arrow* at the extremity of the *gray bar* that represents each clone.

BX278068 and BX278067) originated from the Analysis of Breeding Animals' Genome *Gallus gallus* multitissues cDNA library developed in France by INRA. EST pgr1n.pk002.d11 (EMBL accession number CD217547) originated from the nor-

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TABLE 2
Exon/intron organization of the *OCX-36* gene

No.	Exons			Intron size
	Length	Position in cDNA	Position relative to coding sequence[a]	
	bp			*bp*
1	239	1–239	−127–112	491
2	115	240–354	113–227	95
3	105	355–459	228–332	203
4	156	460–615	333–488	1022
5	64	616–679	489–552	326
6	64	680–743	553–616	336
7	92	744–835	617–708	772
8	174	836–1009	709–882	213
9	54	1010–1063	883–936	244
10	168	1064–1231	937–1104	80
11	68	1232–1299	1105–1172	1185
12	43	1300–1342	1173–1215	400
13	64	1343–1406	1216–1279	178
14	77	1407–1483	1280–1356	532
15	477	1484–1960	1357–1833	

[a] Positions are given according to the cDNA sequence beginning at the ATG initiation codon.

malized chicken reproductive *G. gallus* cDNA library developed by the University of Delaware (26). Finally, EST clone 603791706F1 ChEST756k2 (EMBL accession number BU235026) originated from the *G. gallus* cDNA library developed in Manchester (UK) ((27). The BX273493 and BX273494 (gcal0003c.m.17), BX278067 and BX278068 (gcag 0011c.m.09), and BU235026 ESTs overlapped with the 3′ region of our cDNA, whereas CD217547 allowed the identification of 227 additional nucleotides in the 5′ region, yielding a 1995-bp full-length cDNA sequence (Fig. 2). We named the corresponding protein Ovocalyxin-36 (OCX-36) (see below).

A BlastN search of the entire genome chicken sequence (assembly version of February 2004) was performed. The *OCX-36* gene was located on chromosome 20, between nucleotide positions 9834141 and 9842177. The *OCX-36* genomic sequence is 8037 bp long and consists of 15 exons varying in size from 43 bp (exon 12) to 477 bp (exon 15), with 14 introns that range in size from 80 bp (intron 10) to 1185 bp (intron 11) (Table 2). This chromosome localization was confirmed by genotyping with the ChickRH6 radiation hybrid mapping panel. The genotyping results were submitted to the ChickRH server, and the closest markers were identified by two-point analysis relative to markers already mapped on the ChickRH6 panel. The closest marker, *EXT0064*, was linked to *OCX-36* with a LOD score of 9.69 and at a distance of 19 cR_{6000}. The position of *OCX-36* on the GGA20 RH map can be viewed on the ChickRH server.

Coding Sequence and Amino Acid Translation—The clone, EST, and genomic DNA sequences available were used to generate a general consensus cDNA sequence of 1995 bp (Fig. 3). Within the cDNA sequence, the methionine start codon is at position 128 and the stop codon TGA ends at position 1507 (Fig. 3 and Table 2). The first exon codes for the 5′-untranslated region, the initial 37 codons, and the first base of the 38th codon. The first exon-intron junction is in codon 17 of the mature protein. Exon 15 codes for the seven C-terminal amino acids and the 3′-untranslated region (Table 2).

The translated protein is 459 amino acids in length corresponding to a predicted 48.8-kDa protein. The context of the first start codon at nucleotide 128, with a purine (A) three nucleotides upstream, is compatible with the Kozak consensus sequence for initiation of transcription in eukaryotic species (28, 29). The predicted signal peptide cleavage site is between position 21 and 22 (TQG↓VL) of the translated product. Therefore, the predicted mature N-terminal sequence corresponds exactly to the N terminus obtained by direct amino acid sequencing of the 36-kDa uterine fluid protein (VLSGLSCAIS-PRAMQQVLSDAIIQTG(G)L). The mature protein is therefore predicted to be 438 amino acids long, corresponding to a molecular mass of 46,541 Da. The amino acid composition shows a high content of serine (11.6%) and leucine residues (19.2%), and overall, the hydrophobic amino acid composition is relatively high (43.8%). The internal amino acid sequences obtained by direct sequencing of the tryptic peptides are found in the predicted amino acid sequence obtained from the cDNA sequence. Fragment 2 (VEIYLPR) and fragment 3 (VSLFLXSX-DIG) correspond to positions 418–424 and 392–402 of the OCX-36 amino acid sequence, respectively. Additionally, the 36-kDa band was analyzed by mass spectrometry. MALDI-TOF mass fingerprint and LC-MS/MS analysis allowed the characterization of several peptidic fragments that exactly corresponded to the translated product from the cDNA sequence (Fig. 3). Additionally, peptides derived from Ovocleidin-116 and clusterin were identified by LC-MS/MS in the excised band. We have previously shown these to be eggshell matrix proteins, and we detected OC-116 fragments and clusterin subunits in this molecular weight range by SDS-PAGE (9, 13). The sequences of OCX-36 peptides that were obtained using direct sequencing or mass spectrometry corresponded to the exons 1–3 and 8–13 that cover the N-terminal and C-terminal regions of the translated protein. Forward- and backward-specific primers (36-F3/36-B3) designed for amplification between exons 3 and 8 (nt 283–306 and nt 864–841 of the coding cDNA sequence), respectively, were selected to cover the cDNA sequence coding regions where no amino acid sequence was available (exons 4–7). These primers were used in PCR with reverse transcriptase products from chicken uterine RNA (Fig. 4). The amplicon (about 600 bp) corresponded to the size (582 bp) of the predicted cDNA with exons 4–7. No smaller bands were observed indicating that all exons seem to be expressed in the uterus in a single transcript.

When generating the OCX-36 composite cDNA sequence by alignment of overlapping EST clones, several variations were observed. The BX278067 and BX278068 sequences are the result of 5′ and 3′ sequencing of the gcag0011c.m.09 clone from AGENAE *G. gallus* multitissues cDNA library. These sequences exhibit CGA nucleotides in place of GTT at nt 1489–1491 of the cDNA consensus sequence (nt 1362–1364 of the coding sequence). As a consequence, a glutamic residue is encoded in place of leucine at amino acid position 455, 4 amino acids before the C-terminal end of the protein; the length of the coding sequence remains unchanged. The additional EST (gcal0003c.m.17) from the AGENAE *G. gallus* multitissues cDNA (BX273493 and BX273494 sequences) shows a triplet insertion (CCA) at nt 1419, 1420, and 1421 of the consensus sequence (nt 1292, 1293, and 1294 of the coding sequence). The additional codon results in a 1380-bp coding sequence instead

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of the 1377-bp length of the consensus sequence, which leads to a serine residue replacing glycine at position 432, followed by an arginine. The remaining sequence is identical.

Analysis of Ovocalyxin-36 Protein Sequence—A BlastN search over the entire EMBL data base did not detect any other sequence with significant homology. In contrast, BlastP searches showed significant alignments with mammalian lipid-binding proteins (LBP), bactericidal permeability-increasing proteins (BPI), and cholesterol ester transport proteins and with palate, lung, and nasal epithelium clone (Plunc) family proteins originally observed in mouse palate, nasal epithelium, and adult lung. The predicted amino acid sequence is 20–25% identical to LBP/BPI and to Plunc family proteins. The homology for conservative substitutions ranges from 39 to 44%. The Blast *E*-values between OCX-36 and these proteins range from 10^{-28} to 10^{-4}, indicating a significant relationship between these proteins.

The OCX-36 homology with LBP and BPI proteins is confirmed by data base searching for protein domains and functional sites with the interproscan module from the European Bioinformatics Institute and the eukaryotic linear motif resource for functional sites in proteins. Ovocalyxin-36 protein showed significant matches with the LBP/BPI family with *E*-values ranging from 10^{-21} to 10^{-8}. The region of similarity extends over the entire length of the OCX-36 sequence. Residues 33–244 and 273–439 of the OCX-36 amino acid sequence corresponded to the N-terminal and C-terminal domains, respectively, of the LBP/BPI family proteins. Although a possible glycosaminoglycan attachment site was detected at position 23–26 using the eukaryotic linear motif resource for functional sites in proteins, this post-translational modification was not detected by direct sequencing of this region (Fig. 3). Similarly, predicted phosphorylation sites catalyzed by protein kinase C (Ser-31, -63, and -323) or by casein kinase-2 (Thr-135, 145, and Ser-304) were not detected by peptide sequencing (Fig. 3).

Because of the high composition of hydrophobic amino acids, this feature of OCX-36 sequence was further examined using the method of Kyte and Doolittle (window size = 19), available on line. This analysis revealed zones of extreme hydrophobicity distributed throughout the sequence (Fig. 5).

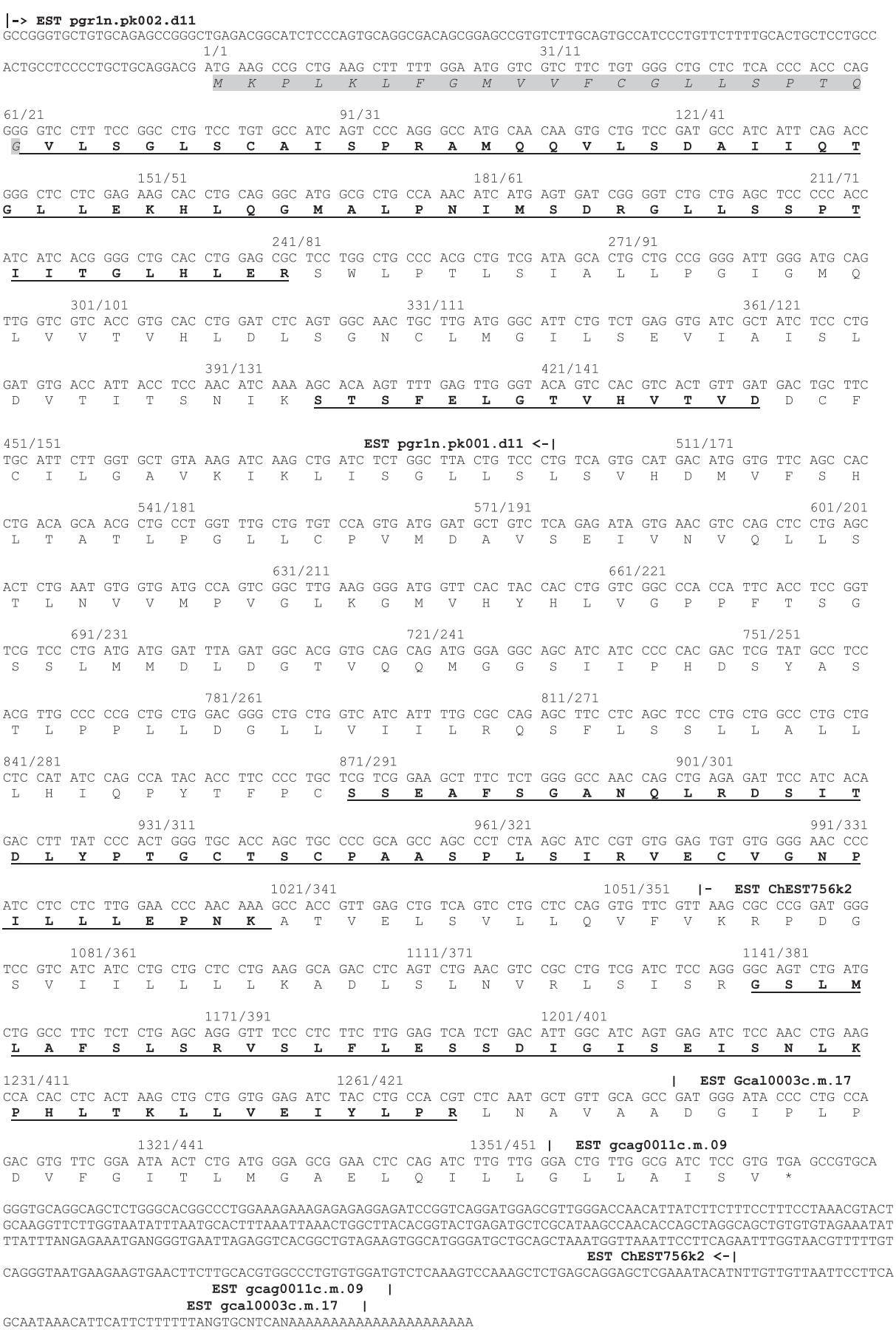
Immunolocalization of Ovocalyxin-36 in the Eggshell—An antibody raised to a synthetic peptide derived from the amino acid sequence (KHLQGMALPNIMSDR, residues 51–65) was used for immunochemistry and Western blotting. The presence of OCX-36 within the uterine fluid and in eggshell extracts was investigated by Western blotting (Fig. 1*b*). A strongly positive immunoreactive 36-kDa band was observed in the uterine fluid collected during the active calcification phase (Fig. 1*b*, *lane 3*). Less intense immunoreactivity was also observed in the initial and terminal stage of shell calcification in uterine fluid (Fig. 1*b*, *lanes 2* and *4*). Additional bands of higher molecular weight were also observed in uterine fluid collected during the active phase where the signal was the most intense, which could correspond to multimers of the immunoreactive band or to post-translational modification of OCX-36 as observed previously for Ovocleidin-116 (13). Prolonged heating of the SDS-treated sample in the presence of β-mercaptoethanol (see "Experimental Procedures") did not diminish the higher molecular weight immunoreactive bands (not shown). The prediction that Ovocalyxin-36 is an eggshell matrix protein was investigated by Western blotting on eggshell extracts in which eggshell membranes were removed from embedded shell using various methods as follows: mechanical scraping (extract 1), chemical treatment using only EDTA (extract 2), or chemical treatment using EDTA and then HCl (extracts 3–4). S.E. examination showed complete removal of the membranes in the samples in which the interior of the shell was further treated with HCl (Fig. 6*b*). For extract 3, although the 5-min HCl treatment removed the vast majority of the membrane fibers, a minor amount of residual fibers remained (data not shown). Demonstration of OCX-36 in the shell compartment was thus determined from the 20-min HCl-treated samples where no residual membrane fibers remained (extract 4). The presence of a 36-kDa immunoreactive band even after mechanical and chemical stripping of membranes revealed that OCX-36 is an eggshell matrix protein (Fig. 1*b*, *lanes 5–8*, *eggshell extracts 1–4*). OCX-36 is present in extracts prepared from shell with membranes (Fig. 1*b*, *lanes 5* and *6*, *extracts 1* and *2*), extract with some remaining residual membranes (Fig. 1*b*, *lane 7*, *extract 3*), and most importantly, in extract containing only calcified part of the shell and no membranes (Fig. 1*b*, *lane 8*, *extract 4*).

The distribution and localization of OCX-36 within decalcified eggshells was investigated by immunofluorescence in order to confirm the Western results. Positive staining was observed throughout the eggshell and in the membranes (Fig. 7). The signal was the most intense at the interface between the membranes and the calcified mammillary cones where mineralization of the shell is initiated. Positive signal at the shell surface was confirmed as OCX-36 immunoreactivity by Western blotting with samples prepared by limited dissolution of the eggshell surface (data not shown).

These observations were further confirmed using colloidal-gold immunolabeling and transmission electron microscopy to reveal ultrastructural localization. Colloidal-gold immunolabeling was observed in both the shell membranes and in the calcified part of the shell, where it was most abundant in the mammillary cone region (Fig. 8*a*). In the inner membranes closest to the egg interior, OCX-36 was present in both the mantle and the core of the fibers (Fig. 8*b*), whereas in the membranes interfacing with the mammillae of the shell, the mantle labeling was lost, and only the fiber cores were positive (Fig. 8*a*).

Tissue Origin and Expression of Ovocalyxin-36—The tissue origin of OCX-36 was evaluated using RT-PCR. The expression of OCX-36 mRNA was measured in various segments of the oviduct involved in the deposition of egg components and in other organs. OCX-36 mRNA expression was only detected in oviduct regions where eggshell calcification takes place (uterus and red isthmus) (Fig. 9). No expression was detected in the proximal oviduct (magnum and white isthmus) or in other organs (liver, kidney, duodenum, and muscle). This pattern of expression was confirmed using quantitative measurements (real time RT-PCR) (data not

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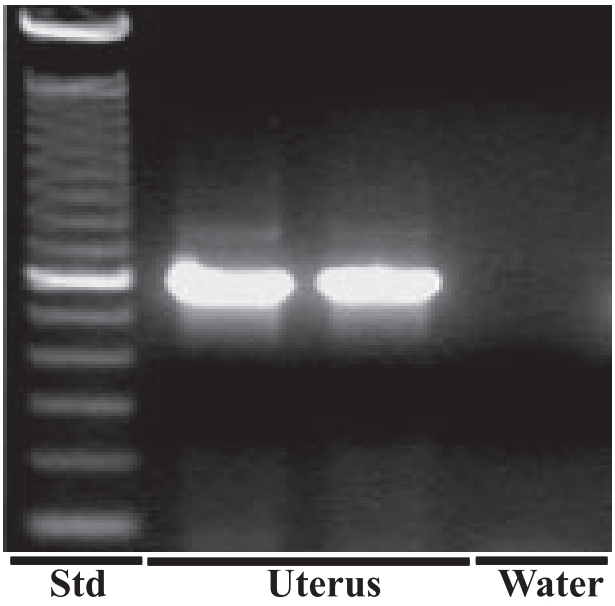


FIGURE 4. **Reverse transcriptase-PCR to detect uterine ovocalyxin mRNA expression of exons 3–8 of the cDNA coding sequence.** Total uterine RNA was extracted from two birds, sampled during eggshell calcification. Following reverse transcription, PCR was performed to amplify a 582-bp portion of the OCX-36 sequence. *Std,* 100-bp DNA ladder.

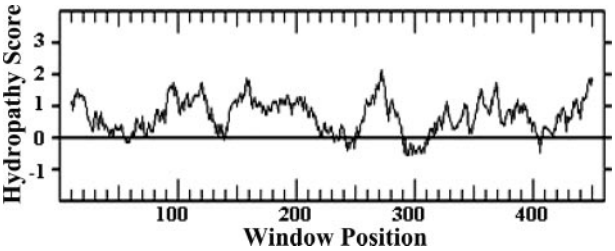


FIGURE 5. **Hydrophobicity plot for Ovocalyxin-36.** Analysis of hydrophobicity using the method of Kyte and Doolittle (window = 19) was conducted using on-line resources.

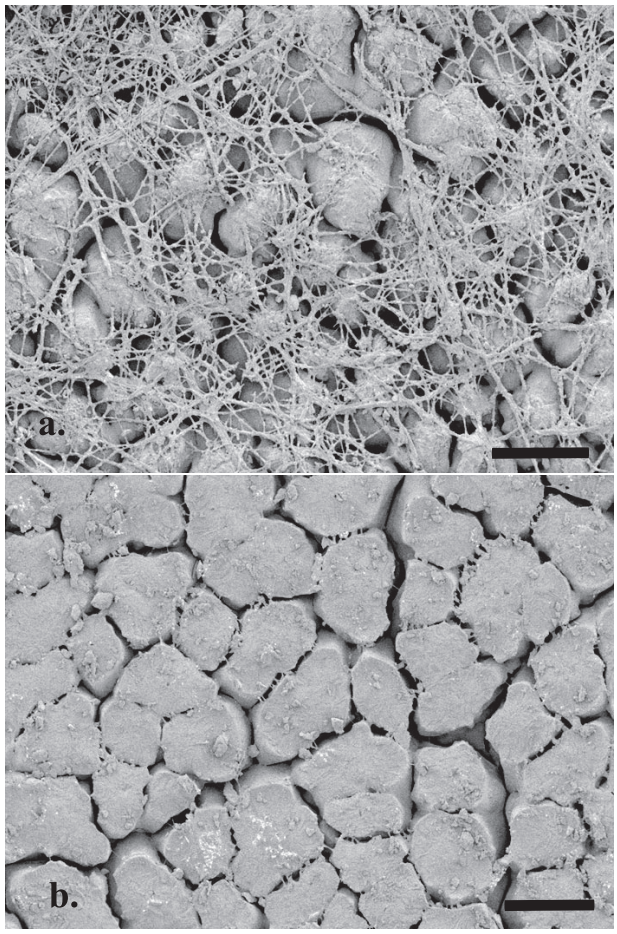


FIGURE 6. **Scanning electron microscopy of the inner shell after chemical treatment to remove eggshell membranes.** Fragments of eggshell were prepared as described under "Experimental Procedures." After mechanical removal of the membranes and incubation of the egg interior with EDTA (*a*), numerous eggshell membranes fibers remained firmly attached to the tips of the mammillary bodies. When the interior of the shell was further treated with HCl (*b*), to partially dissolve mineral from the tips of the mammillary bodies, no membranes fibers were visible at the inner surface of the shell. *Scale bar* equals 100 μm.

shown). The relative levels of OCX-36 message were normalized to 18 S RNA to control for possible differences in RNA extraction and reverse transcription efficiencies between samples. The relative normalized OCX-36 expression was highest in the uterus (0.861 $\pm$ 0.432) and statistically different from all the other tissues tested ($p < 0.001$). In the red isthmus, the expression was 35.7% that expressed in the uterus (0.308 $\pm$ 0.299) and was significantly higher than that detected in all other tissues ($p < 0.001$), where the relative normalized OCX-36 expression was negligible (<0.0008).

The real time RT-PCR technique was also used to determine the basal expression of OCX-36 in uterus from birds with a developing oviduct prior to sexual maturity and accompanying egg production. This group was compared with uterus from mature hens in which the egg was expelled before eggshell deposition to measure the changes in expression induced by sexual maturity and associated hormones. In addition, OCX-36 uterine expression was compared for birds that were sacrificed when there was a hard-shelled egg in the uterus. This last group would reveal any stimulation because of either the dilation induced by presence of the egg or by the process of eggshell calcification and/or hormones involved in regulation of calcium metabolism. The relative expression (ratio of OCX-36/18 S; Fig. 10*a*) was basal in immature uterus (0.0092 $\pm$ 0.003). Sexual maturity stimulated the expression of OCX-36 (0.153 $\pm$ 0.080) to a higher level ($p < 0.0001$) than that observed in the immature uterus. Finally, OCX-36 uterine expression was greatest when an egg was present in the uterus during eggshell calcification (1.51 $\pm$ 0.074, $p < 0.0001$). This considerable stimulation of OCX-36 mRNA expression, when an egg dilated the uterus and was undergoing shell calcification (18 h post-oviposition), was further confirmed by comparison with uterine expression at the stage of egg white deposition when the egg is in the magnum (3 h post-oviposition). OCX-36 mRNA expression (Fig. 10*b*) was 18-fold higher in uterus of hens sampled

FIGURE 3. **Nucleotide and translated amino acid sequence for Ovocalyxin-36.** The full-length composite sequence is derived from expression and hybridization screening results and sequences of overlapping EST clones pgr1n.pk002.d11, chEST756k2, gcal0003c.m.17, and gcag001c.m.09. The putative signal peptide (residues 1–21) is *italic* in *shaded gray*. Residues that have been directly microsequenced are *underlined boldface characters.*

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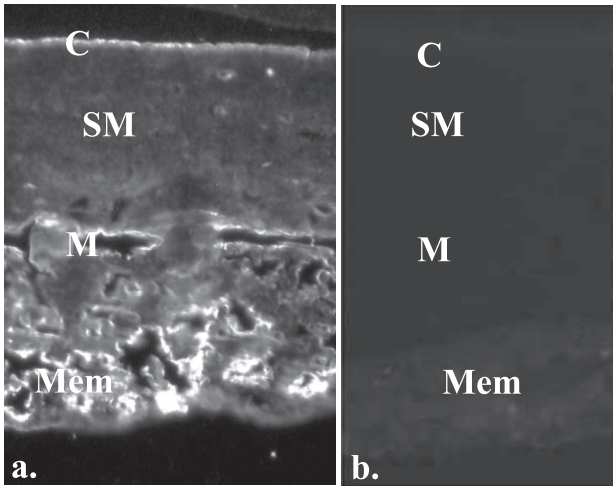


FIGURE 7. **Immunofluorescence localization of Ovocalyxin-36 in decalcified shell.** Normal shell was fixed, decalcified, and paraffin-embedded prior to sectioning (see "Experimental Procedures"). Sections were incubated with Ab Ocx36-(51–65) (1:200) (*a*) or normal rabbit preimmune serum (*b*). *C,* cuticle; *SM*, shell matrix; *M*, mammillae; *Mem*, eggshell membranes.

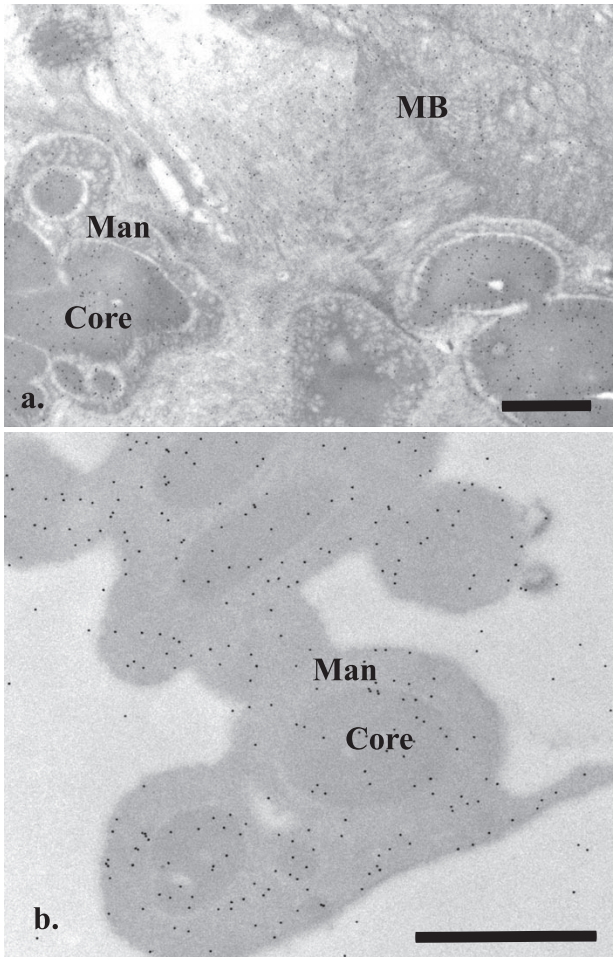


FIGURE 8. **Transmission electron microscopy of eggshell after colloidal-gold immunocytochemistry for Ovocalyxin-36.** Gold particles are observed in the calcified part of the shell (*a*). Labeling was most intense in the basal part of the shell closest to the membranes in which calcified mammillary bodies (*MB*) and eggshell membranes show labeling. The mantles (*Man*) of the outer membranes embedded in the shell are unlabeled. In contrast, within the inner shell membranes that are closer to the egg interior, both mantle and core are labeled (*b*). *Scale bar* equals 1 μm.

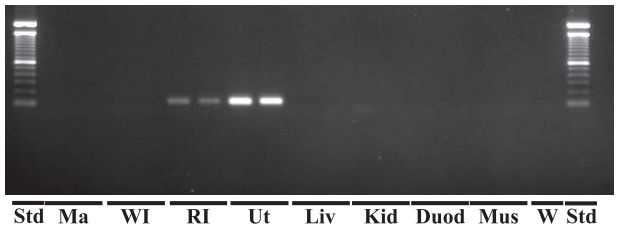


FIGURE 9. **Reverse transcriptase-PCR to detect expression of Ovocalyxin-36 mRNA.** Total RNA from two different birds was extracted from different parts of the oviduct (magnum (*Ma*), white (*WI*), and red isthmus (*RI*), uterus (*Ut*)) and from liver (*Liv*), kidney (*Kid*), duodenum (*Duod*), and muscle (*Mus*) sampled during eggshell calcification. Following reverse transcription, PCR was performed to amplify a 121-bp portion of the OCX-36 sequence. *W*, water; *Std*, 100-bp DNA ladder.

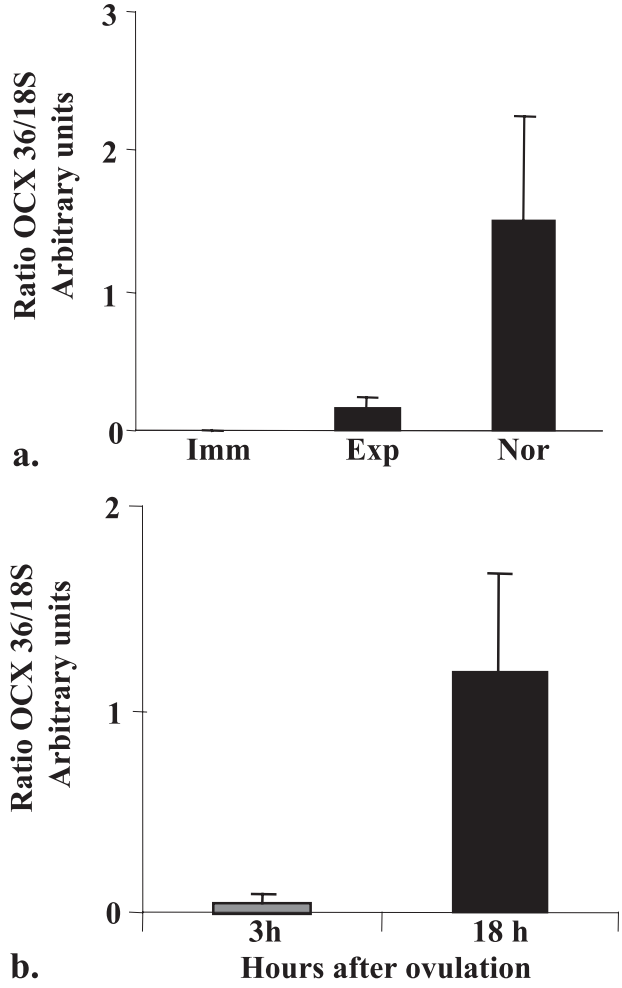


FIGURE 10. **Ovocalyxin-36 mRNA expression pattern in various physiological conditions detected in uterus using quantitative real time RT-PCR.** After reverse transcription, OCX-36 expression levels were normalized to levels of 18 S rRNA extracted from the same tissues (see "Experimental Procedures"). *a,* relative level of OCX-36 expression in uterus from immature (*Imm*) hens, hens with egg expelled (*Exp*) before the calcification of shell, and in hens when a hard-shelled egg is present in uterus (*Nor*). *b,* level of OCX-36 expression detected in uterus when the egg was in magnum during the egg white deposition (3 h after ovulation) and when a hard-shelled egg is forming in uterus (18 h after ovulation).

with a calcified egg undergoing eggshell deposition, compared with empty uterus when no shell was forming and the egg was in the proximal segment of the oviduct (1.15 ± 0.49 *versus* 0.0624 ± 0.0320, $p < 0.0001$).

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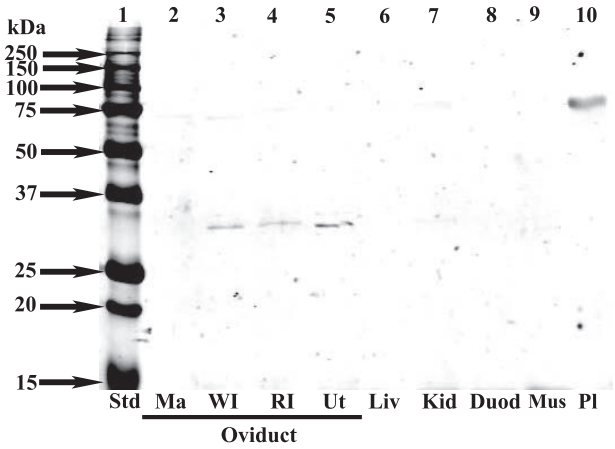


FIGURE 11. **Western blotting to detect OCX-36 in various tissues.** Tissues were harvested from a laying hen at previous oviposition 18 h and processed for Western blotting. *Lane 1*, molecular weight markers (*Std*); *lane 2*, magnum (*Ma*); *lane 3*, white isthmus (*WI*); *lane 4*, red isthmus (*RI*); *lane 5*, uterus (*Ut*); *lane 6*, liver (*Liv*); *lane 7*, kidney (*Kid*); *lane 8*, duodenum (*Duod*); *lane 9*, muscle (*Mus*); *lane 10*, plasma (*Pl*). In tissues sampled at previous oviposition 3 h, only the uterus sample possessed an OCX-36 immunoreactive band.

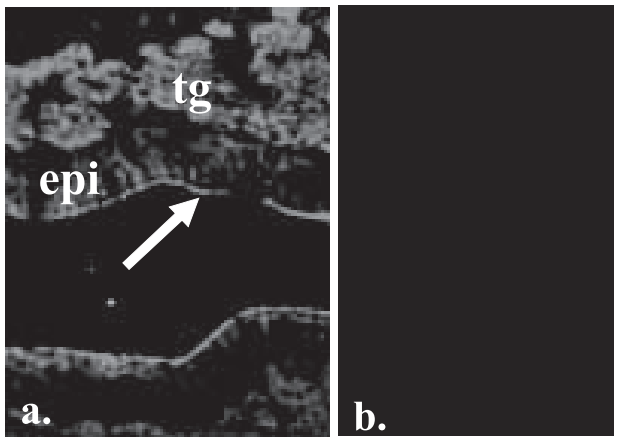


FIGURE 12. **Immunofluorescence microscopy of Ovocalyxin-36 localization in hen uterine tissue.** Thin slices of uterus tissue were fixed and paraffin-embedded (see "Experimental Procedures"). Sections were incubated with Ab Ocx36-(51–65) (1:500) (*a*), or normal rabbit preimmune serum (1:125) (*b*). Strong immunostaining was observed in tubular glands (*tg*), whereas the epithelial cells (*epi*) were mainly negative. The *arrow* indicates the thin line of intense staining seen on the luminal surface of the epithelium.

The presence of OCX-36 protein in other tissues was examined by Western blotting (Fig. 11). The 36-kDa band was not detected in most tissues tested; however, its presence in white isthmus, red isthmus, and uterus was revealed in tissues harvested at 18 h post-oviposition (Fig. 11). Only uterine tissue was positive at 3 h post-oviposition. The 36-kDa band was not detected in magnum (3 and 18 h post-oviposition) nor in egg white (not shown). An unidentified band at 80 kDa was detected in plasma and was occasionally noted in tissue samples.

Finally, the cellular origin of OCX-36 was evaluated by immunofluorescence microscopy. Analysis of uterine tissue revealed a strong immunostaining of tubular gland cells, whereas the epithelial cells were negative (Fig. 12). However, in most sections, a thin line of intense staining was observed on the luminal surface of the epithelium, which could correspond to adherence of secreted OCX-36.

DISCUSSION

The eggshell is a sophisticated structure that is essential for reproduction in all avian species. It regulates the diffusion of water, oxygen, and carbon dioxide between the external environment and the developing embryo. It functions as a mechanical barrier to protect the egg contents from the microbial and physical environment. Therefore, the strength and integrity of the eggshell are critical for survival of the developing embryo.

In this study we report the identification of a novel eggshell-specific protein with an apparent molecular mass, as determined by SDS-PAGE, of 32–36 kDa. This protein was originally identified in earlier studies as a 36-kDa band by SDS-PAGE (2) and was found to be most abundant in uterine fluid during the active phase of calcification. A polyclonal antibody against OCX-36 was used to screen a cDNA expression library prepared with mRNA purified from hen uterine tissue. A positive clone was sequenced and used for further hybridization screening. The resulting consensus sequence was subsequently assembled with ESTs to obtain a complete full-length cDNA. The cDNA and corresponding protein sequence are novel, and it was therefore named Ovocalyxin-36 (OCX-36) (*i.e. Ovum* for egg, *Calyx* for shell, and 36 for its apparent molecular weight).

The conceptual translation product from this cDNA corresponded to a mature protein of 46.5 kDa. The regions of the 36-kDa protein that were sequenced using direct sequencing or mass spectrometry corresponded to exons 1–3 and 8–13 (Fig. 3) that cover both N- and C-terminal regions of the translated protein. Consequently, the notable difference between the molecular weight of the uterine fluid protein and that of the conceptual translated product cannot be explained by a partial degradation of the protein. Because no peptidic fragments matched exons 4–7 and 14, additional mechanisms such as alternative splicing could explain this discrepancy. To check this hypothesis, we designed primers for RT-PCR to specifically amplify, in the uterus, the OCX-36 coding sequence containing the exons that code for the protein sequence for which no peptidic fragments were found. Only one amplicon could be obtained (Fig. 4). Its size (600 bp) indicated that exons 4–7 are expressed in the uterus transcript. No smaller cDNA amplicon was observed. Consequently, we conclude that OCX-36 is expressed as a full-length mRNA with no alternative splicing and that full-length protein synthesis occurs.

Expression screening with the original polyclonal antibody raised to the excised 32–36-kDa band had also identified clones with OC-116 and clusterin inserts, probably because of heterogeneity of the antigen band. Therefore, a more specific antibody to OCX-36 was raised against a synthetic peptide. Western blotting with this antiserum revealed a corresponding immunoreactive band in uterine fluid collected during the initial, active calcification and terminal phases of shell formation (Fig. 1*b*). This protein was particularly abundant in uterine fluid harvested during the active calcification phase, where a "ladder-like" series of immunoreactive bands was revealed. Because this pattern was resistant to prolonged heating in the presence of β-mercaptoethanol, we hypothesize that OCX-36 is susceptible to cross-linking such as that catalyzed by lysyl oxidase in the

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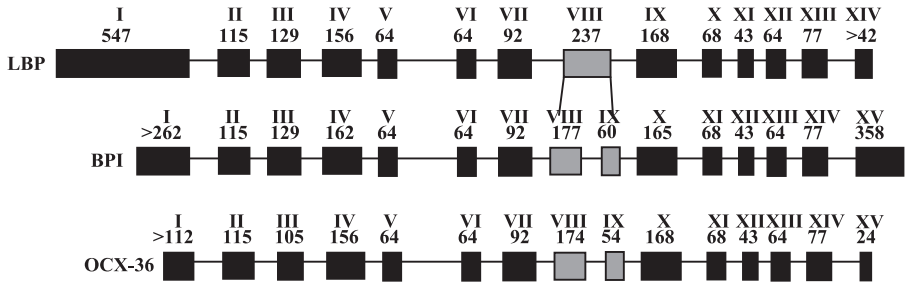


FIGURE 13. **Comparison of the genomic structure of human LBP, human BPI, and OCX-36 proteins.** Exons (*roman numbers*) and length (*arabic numbers*) are indicated. Conserved exon lengths are emphasized by *black boxes*. Introns are not drawn to scale. Human BPI, accession number NM_001725; human LBP, accession number NM_004139.

white isthmus of the hen oviduct (30). Alternatively, oligomerization of OCX-36 under conditions of SDS-PAGE is due to its highly hydrophobic amino acid sequence, as has been demonstrated for α-zein (31). Either cross-linking (intramolecular) or abnormal behavior in SDS-PAGE because of hydrophobicity would provide an explanation for the unusual discrepancy between the sequence molecular weight and that detected by SDS-PAGE, as noted in the previous paragraph. Ongoing studies to purify OCX-36 protein to homogeneity will allow further characterization of the protein.

Western blotting for OCX-36 reveals its presence in decalcified eggshell extract (Fig. 1*b*), suggesting that it becomes incorporated into the calcified shell. This was confirmed by various treatments designed to obtain shell extracts with no remaining membrane organic fibers. When the eggshell membranes were mechanically removed or only treated with EDTA, organic fibers remained firmly attached on the calcified part of the shell (Fig. 6*a*). When the shell was treated with EDTA, followed by HCl, mammillary bodies were more fully decalcified, and the membranes could be completely removed from the calcified part of the shell (Fig. 6*b*). An OCX-36 immunoband was seen in extracts treated with HCl (Fig. 1*b*, *lanes 7* and *8*) and consequently lacking any remaining membranes, demonstrating that OCX-36 is also a matrix protein of the calcified shell. This was confirmed by immunolocalization studies at both the light and electron microscopic levels, where OCX-36 was detected throughout the eggshell, with the strongest immunostaining in the inner region of the shell adjacent to the membranes (the mammillary cone layer). This localization of Ovocalyxin-36 is in agreement with the recent proteomic analysis of the acid-soluble organic matrix of the chicken eggshell in which OCX-36 was identified as a protein constituent of the calcified matrix (32). Three other eggshell-specific proteins have been localized in the mineralized region of the eggshell. Ovocalyxin-32 is most abundant in the outer shell (16), whereas Ovocleidin-116 is most intensively detected in the palisade layer (13). On the other hand, Ovocleidin-17 is abundantly distributed throughout the mammillary cones and the palisade layer (10).

The tissue expression of OCX-36 was investigated using RT-PCR and subsequently quantified using real time RT-PCR. OCX-36 message was only observed in the tissue where eggshell calcification takes place (Fig. 9). No expression could be detected in the other segments of the oviduct or in any other tissue tested. However, OCX-36 protein was detected in white isthmus, red isthmus, and uterus (Fig. 11). This suggests that transfer of OCX-36 protein within the lumen of the oviduct occurs from its distal origin (red isthmus and uterus) toward the white isthmus. OCX-36 migration in this manner would account for its association with the shell membranes.

Two types of secretory uterine cells, those of the surface epithelium and of the underlying tubular glands, are involved in secretion of eggshell matrix proteins. The proteoglycans Ovocleidin-116 and Ovocalyxin-32 are secreted by the surface epithelium (13, 16). In contrast OCX-36 is secreted by tubular gland cells (Fig. 12) as already reported for Ovocleidin-17, ovotransferrin, and clusterin (5, 9, 10).

Physiological regulation of *OCX-36* gene expression during egg formation was investigated by quantitative mRNA expression studies (Fig. 10). OCX-36 mRNA was highly expressed in the uterus of sexually mature birds with a calcifying eggshell, compared with expression before entry of the egg and initiation of calcification. Similarly, the expression of osteopontin into the hen uterus is up-regulated by the entry of the egg into the uterus (33). This enhanced expression is attributed to the mechanical distension exerted upon the uterine wall. Uterine expression of a heparan sulfate proteoglycan, parathyroid hormone-related protein, and Na^+-K^+-ATPase is up-regulated by the same mechanisms (34–36).

We propose an antimicrobial role for OCX-36 protein because its protein sequence has significant similarity with LBP, BPI, and Plunc family proteins. These proteins are well known in mammals for their involvement in defense against bacteria. They belong to the superfamily of proteins known to be key components of the innate immune system, which act as the first line of host defense (37). Homology between OCX-36 and the LBP/BPI family of mammalian proteins is further reinforced by comparison of their gene structure. The exon/intron organization of the *OCX-36* gene is very similar to that of the highly conserved *LBP* and *BPI* genes (38), with most corresponding exons possessing identical sizes (Fig. 13). Furthermore, *OCX-36* maps to GGA20, which presents a conservation of synteny with the portion of HSA20 containing *LBP* and *BPI*. These data strongly suggest a common origin for *LBP*, *BPI*, and *OCX-36*, with a recent duplication in the mammalian lineage and rapid nucleotide divergence leading to the low similarity at the DNA level between *OCX-36* on one side and *LBP/BPI* on the other.

BPI is composed of N-terminal and C-terminal domains (39). Using the eukaryotic linear motif resource for functional sites in proteins, we observed that positions 33–244 and 273–439 of the OCX-36 protein sequence matched the BPI1 N-terminal and BPI2 C-terminal domains of BPI proteins, respectively. Therefore, OCX-36 is predicted to possess similar overall structure and protein folding as seen in the human BPI crystal structure (40). Another chicken BPI analog, Tenp (18% identity with human BPI), was shown by proteomic analysis to be a hen

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egg white component (41). Surprisingly, Blast 2 sequence comparison revealed that Tenp and OCX-36 amino acid sequences have only 18% identity with each other (homology climbs to 39% for conservative replacements). OCX-36 and Tenp are therefore different egg proteins with significant sequence similarity to BPI proteins.

Therefore, we hypothesize that OCX-36 contributes to natural egg defenses by providing chemical protection for the egg contents, particularly in the lumen of the distal oviduct during its formation in the white isthmus, red isthmus, and uterus. Although the chicken eggshell is a very effective protective system, bacteria sometimes penetrate the egg or could be already present in the uterus via retrograde movement from the cloaca before eggshell formation. Egg albumen presents an efficient, natural barrier to bacterial contamination of the egg contents. Antimicrobial protection is a function that has been most commonly ascribed to numerous egg white proteins that possess antimicrobial properties (19). Lysozyme hydrolyzes the bacterial peptidoglycan resulting in cell wall lysis of Gram-positive bacteria. Ovotransferrin inhibits Gram-negative bacteria by sequestering iron, which is essential for their growth. We have demonstrated that these two major components of egg white are also eggshell matrix proteins (4, 5) and may also influence the calcification process. However, they would also function as anti-bacterial agents in the eggshell and its membranes. Moreover, partially purified eggshell matrix exhibits antimicrobial activities against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus cereus*, which cannot be solely explained by the presence of lysozyme or ovotransferrin (42). Antimicrobial protection may involve a synergistic effect with additional antibacterial proteins such as OCX-36. In this study we have shown that OCX-36 is most abundant in the basal shell and associated membranes. Eggshell dissolution from the calcium reserve body at the bases of the mammillary bodies occurs prior to hatching in response to acid secreted by the chorioallantoic membrane. This process would solubilize OCX-36 and up-regulate antimicrobial protection as the eggshell becomes progressively weaker in preparation for hatching.

In conclusion, OCX-36 is a novel eggshell protein that is secreted during shell formation and is found in the lumen of the distal oviduct, becoming incorporated into the eggshell membranes and the eggshell. OCX-36 may be involved in the innate defense of the egg to keep the egg free of pathogens and safe for the developing embryo and, fortuitously, for the human consumer.

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