The upregulation of messenger ribonucleic acids during 17 α ,20 β -dihydroxy-4-pregnen-3-one-induced ovulation in the perch ovary

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ABSTRACT

While progestins appear to be involved in the local ovarian regulation of vertebrate ovulation, their specific role is unclear. In yellow perch (*Perca flavescens*) the progestin, 17 α ,20 β -dihydroxy-4-pregnen-3-one (17,20-P), stimulates ovulation *in vitro* and this induction requires gene activation. Therefore, the perch model was used to isolate progestin-upregulated mRNAs. Perch ovaries were incubated for 32 h with or without 17,20-P (0.1 μ g/ml). Messenger ribonucleic acids were isolated from the tissue and used for differential display PCR (DDPCR). From DDPCR, 5 bands were eventually obtained that were verified by Northern analysis to be consistently upregulated by 17,20-P at 32 h. Using these bands, full-length cDNAs were obtained by library screening and completely sequenced. Based on similarity to known sequences, four of the cDNAs presumably encode for perch forms of (1) neprilysin (PNEP-1; 63% identical); (2) a lysyl oxidase-type protein (PLO-2; 43.2% identical); (3) calmodulin (PCAL-1; 100% identical); and (4) a microtubule aggregate-like

protein (PMAP-1; 29.6% identical). The fifth cDNA obtained from DDPCR most likely encodes for an egg protein and will be reported separately. Each of the cDNAs was used to probe Northern blots of ovarian mRNA taken at 0, 12, 24, 32 and 42 h of incubation with 17,20-P. This temporal Northern analysis verified that all four were upregulated by 32 h. In addition, PNEP and PMAP transcripts began to increase by 12 h, while PCAL and PLO transcripts remained elevated through 42 h. On Northern blots of RNA from other perch tissues, calmodulin was found in all tissues, PLO mRNA was ovarian specific, and PMAP mRNA was also present in the gills and liver. Multiple transcripts were observed for PNEP, but the ovarian form induced by 17,20-P was only found in high abundance in the heart. To our knowledge, this is the first report that specifically characterizes progestin upregulated mRNAs in the vertebrate ovary at ovulation.

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INTRODUCTION

Through the results of a number of diverse studies, progestins have been implicated in the local ovarian control of vertebrate ovulation. For example, spontaneous or gonadotropin-induced ovulation in rats can be blocked by steroid synthesis inhibitors such as aminoglutethimide or cyanoketone (Lipner & Greep 1971, Lipner & Wendelken 1971). Further, progesterone antiserum and antiprogestins also block human chorionic gonadotropin (hCG)-induced ovulation (Mori *et al.* 1977, Donath *et al.* 1997). In some investigations, the inhibition of ovulation could be restored with exogenous progesterone (Mori *et al.* 1977), although this has not

always been the case (Lipner & Greep 1971, Lipner & Wendelken 1971). In certain studies the ineffectiveness of exogenous progesterone replacement may have been a result of the precise timing of the application of progesterone relative to the antagonist. For example, it was demonstrated that hypophysectomy blocked ovulation in rats and that progesterone could reverse this inhibition (Takahashi *et al.* 1974). However, the timing and the duration of exogenous progesterone treatment was critical in restoring ovulation in hypophysectomized animals (Takahashi *et al.* 1974).

Perhaps the most convincing evidence for a progestational role in ovulation has come from studies using *in vitro* perfused ovaries. In the

perfused rat ovary, inhibition of 3β -hydroxysteroid dehydrogenase blocked luteinizing hormone (LH)-induced ovulation, and this could be restored by progesterone (Brannstrom & Janson 1989). Progesterone can also stimulate ovulation by itself in the perfused fowl ovary, indicating a direct action of progestins in the bird ovary (Tanaka *et al.* 1987). However, in several studies using the perfused rabbit ovary, inhibitors of steroidogenesis were unable to block gonadotropin-induced ovulation (Holmes *et al.* 1985, Yoshimura *et al.* 1986, 1987), indicating that progestins may not be involved in the control of ovulation in all vertebrates.

Finally, mice carrying a null mutation of the progesterone receptor are anovulatory even though they can undergo follicular development to produce preovulatory follicles (Lydon *et al.* 1995). Further, exogenous gonadotropin treatment will not stimulate ovulation in these mutants.

While progestins appear to be involved in the local ovarian regulation of ovulation, their specific role is unclear. In the ewe, the inhibition of steroidogenesis by isoxazol blocks ovulation and prostaglandin (PG) F production in the ovary (Murdoch *et al.* 1986). It has been hypothesized that progesterone stimulates the conversion of PGE to PGF in the ewe by stimulating the enzyme PGE_2 -9-ketoreductase, thereby increasing the levels of PGF at ovulation. In support of this, isoxazol decreases the activity of PGE_2 -9-ketoreductase while progesterone restores this activity (Murdoch & Farris 1988). Thus, one role of progestins in the ovary may be to regulate prostaglandin synthesis. Progesterone inhibits the expression of prostaglandin endoperoxide synthase 2 (PGS-2) in the LH-stimulated rat ovary (Hedin & Eriksson 1997). Since inhibition of PGS-2 would lead to a decrease in prostaglandin synthesis, this particular effect of progesterone would appear to be opposite to the stimulatory role implied by the results of earlier investigations. However, it has been hypothesized that one function of progesterone may be to restrict the inflammatory response at the time of ovulation (Hedin & Eriksson 1997). In addition, past inconsistencies in the effects of progesterone on ovulation may result from the possibility that progesterone has both stimulatory and inhibitory actions at ovulation depending on the precise time that it acts.

Besides eicosanoid synthesis, progestins have also been implicated in the ovarian stimulation of metalloproteinase inhibitors (Morgan *et al.* 1994), plasminogen activator (Tsafiri *et al.* 1987), poly(ADP-ribose) polymerase (Murdoch 1998) and the kallikrein/kinin system (Tanaka *et al.* 1992).

In yellow perch (*Perca flavescens*) the progestin, $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (17,20-P), stimulates both final oocyte maturation (resumption

of meiosis) and ovulation completely *in vitro* (Goetz & Theofan 1979). As a result, this organism has proven to be a very useful model in which to study the regulation of ovulation. Ovulation induced by 17,20-P can be blocked by indomethacin (Bradley & Goetz 1994), an inhibitor of prostaglandin endoperoxide synthase, and it can be restored by primary prostaglandins (Goetz & Theofan 1979). Thus, in perch it is hypothesized that at least one action of 17,20-P on ovulation requires prostaglandins. More importantly, 17,20-P-induced ovulation can be blocked by actinomycin (Theofan & Goetz 1981), indicating that mRNA transcription is necessary. In the current study, differential display PCR (DDPCR) was used to isolate mRNA transcripts upregulated by 17,20-P in the perch ovary just prior to *in vitro* ovulation. During this study, five progestin upregulated cDNAs were isolated. Characterization of four of the cDNAs is presented in this paper. The remaining cDNA will be reported separately since it is likely that it represents an egg protein of unknown function. To our knowledge, this is the first report that specifically describes progestin-upregulated mRNAs in the vertebrate ovary at ovulation. In addition, two of the cDNAs reported here encode for proteins that have never been associated with the vertebrate ovary or the process of ovulation.

MATERIALS AND METHODS

Animals and ovarian tissue collection

Mature yellow perch females were obtained by hook and line from January to March, or were purchased in March from commercial suppliers in Madison, WI, USA. Fish were held under natural photoperiods in 300 gallon tanks in running well-water at 4 °C. All fish used for *in vitro* incubations were primed approximately 3 days prior to use with 25 IU hCG to initiate germinal vesicle (GV) migration. To follow GV migration accurately, eggs were sampled daily by inserting a 100 μ l glass pipette into the ovipore and removing 5–10 eggs. The eggs were examined microscopically and when the lipid droplets began to coalesce and the GV moved slightly off-center, fish were over-anesthetized in 2-phenoxyethanol and decapitated. Ovaries were removed and placed in ice-cold Cortland medium (Wolf & Quimby 1969) containing HEPES (pH 7.8).

In vitro incubations for differential display PCR and temporal Northern analysis

For large scale *in vitro* incubations to obtain mRNA for DDPCR, the ovary was first dissected into large

sheets (100–300 follicles) to ensure that all follicles were exposed to the medium. These pieces were then divided into two equal portions based on weight. Each portion was transferred to a 1.0 l flask containing 200 ml Cortland medium and either 17,20-P (0.1 µg/ml) or a 95% ethanol vehicle (1.0 µl/ml). The 17,20-P was initially dissolved in 95% ethanol at high concentration and added to the medium to obtain the final steroid concentration. Flasks were incubated at 15 °C under intermittent agitation for 32 h.

For the temporal analysis of mRNA expression for specific upregulated cDNAs, smaller scale incubations were conducted. For these incubations, fish were primed and the ovaries dissected into large sheets as described above. The sheets from each individual ovary were then divided randomly into nine treatment groups (2–3 mg tissue/treatment) and each group was incubated in 50 ml Cortland medium at 15 °C with 17,20-P (0.1 µg/ml) or ethanol control (1.0 µl/ml). Control and 17,20-P-treated samples were then assayed at 12, 24, 32, and 42 h of incubation. In addition, an untreated zero hour sample was also obtained.

RNA isolation

Following *in vitro* incubations, ovarian tissues were deyolked by pressing them between two fine mesh stainless steel screens (198 perforations/cm²). Ice-cold Cortland medium was applied to wash the samples while the screens were gently pressed together. This procedure was used to separate the extrafollicular tissue and follicle layers from the chorion components and egg yolk of mature oocytes. Tissues were scraped into beakers under a stream of ice-cold Cortland medium and then distributed to 50 ml polypropylene tubes. Samples were lightly vortexed, spun at 3000 r.p.m. for 5 min at 4 °C, and the supernatant was decanted into new 50 ml tubes held in ice. The remaining tissue pellet was resuspended in 30 ml new Cortland medium, vortexed, and spun again under the same conditions. This procedure was repeated twice. The supernatants from the second and third washes were removed and discarded, and the tissue pellet remaining after the last wash was homogenized in 3 ml Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) with a TissueTearor (Biospec, Bartlesville, OK, USA). The supernatant remaining after the first wash was centrifuged at 10 000 r.p.m. for 5 min at 4 °C. The supernatant was decanted and the small tissue pellet that remained was resuspended in 500 µl Tri-Reagent. The tissue homogenates were then combined and briefly homogenized. All samples were then

stored at –70 °C until RNA extractions could be completed.

RNA isolation was completed as described previously (Chomczynski & Sacchi 1987, Chomczynski 1993). Briefly, tubes were thawed at room temperature and chloroform was added to the samples (0.2 ml chloroform/1.0 ml Tri-Reagent). The samples were shaken vigorously and then held at room temperature for 15 min. Following a 15 min centrifugation at 12 000 *g* the colorless upper phase containing the RNA was transferred to a new 15 ml RNase-free tube and precipitated for 10 min with isopropanol (0.5 ml/1.0 ml of initial Tri-Reagent volume). Samples were then centrifuged at 12 000 *g* for 20 min and the supernatant was decanted. The RNA pellet was washed twice in 6 ml 75% ethanol and dried for 10 min at room temperature. The RNA pellet was resuspended in 500 µl RNase-free water and held at –70 °C until mRNA isolation was performed using the Poly-A-Tract mRNA Isolation System (Promega, Madison, WI, USA).

Differential display PCR (DDPCR)

The mRNA from large scale ovarian incubations on five female perch was used for DDPCR analysis (RNAmapp; GenHunter Corp., Nashville, TN, USA). For each sample (i.e. control and 17,20-P treatments/ovary) cDNA was made from purified mRNA (0.1 µg) using Superscript II reverse transcriptase (GibcoBRL Life Technologies, Grand Island, NY, USA) and either G or C anchor primers (T12MG or T12MC; GenHunter Corp.). The resulting cDNA was then subjected to PCR using the same oligo dT anchor primers and a random 10 bp primer (AP1 through AP15; GenHunter Corp.) in the presence of [α -³²P]dATP (2000 Ci/mmol; DuPont NEN, Boston, MA, USA). Labeled products were separated on 6% denaturing polyacrylamide gels (32.5 cm by 50.0 cm by 0.4 mm) at 2500 V for 5 h. Gels were then blotted without fixing and exposed to x-ray film for 2 days. Following alignment of the x-ray film and the original gel blot, differential bands were excised using a razor blade.

Gel pieces were soaked in 100 µl water and boiled for 20 min in 2.0 ml microcentrifuge tubes. The samples were spun in a microcentrifuge and the supernatant was transferred to a new 2.0 ml tube. The DNA was precipitated in the presence of glycogen using 3 M sodium acetate and absolute ethanol. The samples were then centrifuged for 10 min at 13 000 *g* to pellet the DNA. Pelleted DNA was washed in 85% ethanol, dried for 10 min, and then resuspended in 10 µl water. This DNA was reamplified by PCR using the same primer

pairs used to generate the original DDPCR bands. Reamplified products were separated on agarose gels, and bands of interest were excised and gel extracted (Qiaex II; Qiagen, Chatsworth, CA, USA). Gel purified DNA was ligated into pCRII (In Vitrogen, San Diego, CA, USA) and used to transform *E. coli* INVaF' cells (Original TA Cloning Kit, In Vitrogen). Positive colonies were screened for inserts using PCR. Of the colonies that contained inserts of the appropriate size DNA, three clones per original DDPCR band were selected for sequencing. Sequencing was completed on plasmid DNA using a modified dideoxy chain termination method (Cy5 AutoRead Sequencing kit; Pharmacia Biotech, Madison, WI, USA) with Cy5-labeled vector primers flanking the DNA insertion site. The sequencing reactions were separated and analyzed using an ALFexpress Sequencer (Pharmacia Biotech). If the sequences of all 3 clones were identical, the DNA of one of the clones was used as a probe for Northern analysis to verify that the transcript was upregulated as observed in the original DDPCR.

Northern analysis

Several types of Northern analysis were completed. Initial verification of 17,20-P upregulation at 32 h was completed on ovarian mRNA from five to seven females depending on the clone. Secondly, Northern analysis was used to determine the exact temporal expression pattern of verified 17,20-P upregulated clones using mRNA extracted from 2–4 individual fish. Finally, Northern analysis was completed to determine the general tissue expression pattern of the four upregulated mRNA transcripts obtained from the DDPCR screen. All Northern analyses were performed in the same manner as described below.

Messenger ribonucleic acid (0.5 µg/lane) was separated on formaldehyde-agarose gels (1.6% agarose, 2.2 M formaldehyde, and 1 × MOPS (3-(*N*-morpholino) propanesulfonic acid)). Samples were mixed 1:1 with a reaction/loading buffer containing 4.2 M formaldehyde, bromophenol blue (0.005 mg/ml), 10% glycerol, and 2 × MOPS. Samples were heated at 55 °C for 15 min prior to loading. Gels were run at 55 V until the dye front had migrated 8 cm. After washing, mRNA was transferred to nylon membranes (Magna Charge Nylon Transfer Membranes, Micron Separations Inc., Westborough, MA, USA) by downward capillary elution using 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.2). After washing in 2 × SSC, membranes were cross-linked (Stratalinker; Stratagene, La Jolla, CA, USA) and stored under vacuum.

Nylon membranes were pre-hybridized for at least 2 h in roller tubes at 42 °C in a buffer containing 5 × SSPE (0.75 M NaCl, 0.05 M sodium phosphate monobasic, 5 mM EDTA, pH 7.4), 0.1% SDS, 5 × Denhardt's solution, 50% formamide, and 150 µg/ml calf thymus DNA. The Northern blots were probed with a radiolabeled, double stranded insert obtained either from EcoRI digests of pCRII products obtained from DDPCR plasmid preps or from XhoI/EcoRI digests of full-length clones obtained from cDNA library screening (see below). Inserts were labeled with [α -³²P]dATP (3000 Ci/mmol; ICN, Irvine, CA, USA) using Klenow and random primers (Prime-It II, Stratagene). The labeled probe was purified using a gel filtration spin column (CENTRI-SEP; Princeton Separations, Adelphia, NJ, USA) and heat denatured in boiling water for 5 min. The denatured probe was added directly to the pre-hybridization buffer and incubated with the Northern blot overnight. Following hybridization, the blots were washed twice (15 min each) under medium stringency (1 × SSPE, 0.1% SDS, 45 °C) and twice (15 min each) under high stringency (0.1% SSPE, 0.5% SDS, 65 °C). Northern blots were dried, exposed to phosphor-imaging screens, and analyzed using a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). For the initial Northern verification of 17,20-P upregulation at 32 h of incubation, RNA bands were quantified using Imagequant (Molecular Dynamics) and analyzed statistically using pair-wise *t*-tests (Zar 1996).

Full-length cDNAs

DNA bands produced by DDPCR represent only a small 3' region of the transcript; thus, complete cDNAs were obtained by library screening. A cDNA library was constructed in Unizap (Zap Express, Stratagene) using pooled mRNA from perch ovaries stimulated for 32 h with 17,20-P. The libraries were plated at 20 000 to 50 000 plaques per plate (12 cm diameter) and lifted to nylon membranes (Magna Charge Nylon Transfer Membranes, Micron Separations Inc.). Lifts were screened using α -³²P-labeled DDPCR inserts as described above (see Northern analysis section). Blots were hybridized and washed exactly as indicated above for Northern blotting and were exposed to X-ray film for two days. Positive plaques were rescreened once to homogeneity. Plaques were excised (Rapid Excision Kit, Stratagene) and the resulting bacterial colonies were verified for inserts using colony PCR. Plasmid DNA from clones of interest were either (1) fully sequenced with internal Cy-5-labeled primers (Thermo Sequenase

1 CGG CAG CAG GTG TCG CGG TGT GTT TGT GTG CAT GTG CGC TCG TAG GAT AGA AAG CCA AAG
61 GAA GAA GAA TAA GGA GGA GAG AGG CGG GAG CGG TGT GTG CGA TGT TAA AAT CCA GGT TGA
121 AGC AAA TAG AGA GTG TGT GAG AGA TCG ATC AGC ACT TCG CGC TCT TGT GAA TTT GAT
181 GCA TCA GCT GAT GTG TGT GAG AGA TCG ATC AGC ACT TCG CGC TCT TGT GAA TTT GAT
Met Pro Ile Tyr Ile Ile Asp Arg Lys Phe Pro Asp Thr Ser Gly
241 GAG TTG ATA CAG CGC GCA GCT GAA GCA GCA GAC CTG AGA ATG ATG GAA ACC AAC CCC CCC
Glu Leu Ile Glu Phe Ala Ala Glu Ala Gly Asp Leu Arg Met Met Glu Thr Asn Pro Pro
301 AAG TCA GCT AAG AAG CCC CGC TCG ACC TCC CTG GAG GTG GGC CTA ACT ACT ATC GTC TCC
Lys Ser Ala Lys Lys Phe Pro Arg Trp Thr Ser Ser Leu Glu Val Gly Leu Thr Thr Ile Val Ser
361 TGG CTC TTT ATC GTC ATC GTC GGC CTC ATC ATC CTC TTT GGC ACA CAG ANG ACT GAT GAA
Tyr Cys Thr Phe Ile Val Ile Val Ala Leu Ile Ile Leu Phe Ala Thr Glu Lys Thr Asp Glu
421 ATC TGC ACC AAG GGT GAC TCG ACA CAA TCA GCG TCT CCG CTC ATT GAG AAC ATG GAT GAT
Ile Cys Thr Thr Gly Asp Cys Thr Glu Ser Ala Ser Arg Leu Ile Glu Asn Met Asp Asp
481 AGT GTG GAC CCT TGT GAG AAC TCT TAC CAG TAC CAG TAC CAG TAC CAG TAC CAG TAC CAG
Ser Val Asp Pro Cys Asp Asn Phe Tyr Glu Tyr Ala Cys Gly Gly Trp Leu Lys Lys Asn
541 ATC ATC CAG GAC ACC AGC TCT AGA TAC AGC ACC TTT GAC ATC TTA CGA GAC GAA CTG GAG
Ile Ile Pro Glu Thr Ser Ser Arg Tyr Ser Thr Phe Asp Ile Leu Arg Asp Leu Glu
601 GTT ATC CTC AAA GGT GTC CTT GAA AAA ACA GAC GAG GGG GAA GCA ACG CTC TCA ACA AGG
Val Ile Leu Lys Lys Val Leu Leu Lys Thr Asp Glu Gly Glu Ala Thr Leu Ser Pro Pro
661 CTT AAG AAC CTT TAC GAG TCC TGT ACC AAT GAG AGT TTA ATT GAG CTC AGG GGA GGC GCT
Ala Lys Thr Leu Thr Lys Ser Cys Thr Asn Glu Ser Leu Ile Glu Leu Arg Gly Gly Ala
721 CTT TCG CTC GAC ATC GTC CTT GAT TTT GAG TGG CCC GTC GTG GAC AAC TGG GAG
Pro Leu Leu Asp Asp Met Leu Pro Asp Val Phe Glu Trp Pro Val Ala Val Asp Asn Trp Glu
781 ACC AAC TAT GGT AAT ACA TGG AAG TGG GAG GAC GTC ATC GGC AAG CTG AAT GAG AAA TAT
Thr Asn Tyr Gly Lys Thr Thr Trp Arg Leu Glu Leu Asp Val Ile Ala Lys Leu Asn Glu Lys Tyr
841 GGA ACT CAA CTC TGT GTC AAC TTT TTT GTC GGC ACC GAT GAC AGG GAC TCC AAT TCA TAC
Gly Thr Glu Leu Leu Val Asn Phe Phe Val Gly Thr Asp Asp Arg Asp Ser Asn Ser Tyr
901 ATC AAT GAT TTT GAC CAG CAG ACA AAC CTC GGC CTC TTA TCT AGA GAT GAT TAC GCT TCG
Ile Ile His Phe Asp Glu Glu Thr Asn Leu Gly Leu Leu Ser Ser Asp Tyr Tyr Ala Cys
961 ACT GGA CCC TAC GCA GAG GCA TGT GGC GGC TAC GAG AAG TTC ATC ATT GAC CTT GGC AAG
Thr Gly Pro Tyr Ala Glu Ala Cys Arg Ala Tyr Gly Lys Phe Met Ile Asp Leu Ala Lys
1021 CTG ATC CGC ATT GAC CGG CGA TGG AAC ATC AGT GAA ACA GAT AGA GAG GAG GTG AAG
Leu Ile Arg Ile Asp Asp Arg Gly Leu Thr Asp Ile Arg Glu Glu Val Lys
1081 CGA GTT ATG CAG CTC GAG AGA CAG ATC GGC AAT GCT ACC AAT ACT CCA GAG CAG COT AAC
Arg Val Met Asp Leu Glu Arg Asp Ile Ala Asn Ala Thr Asp Thr Pro Glu Asp Arg Asn
1141 AAC CCA GTG TTT TTT TAC AAC AAG ATG GAA CTG GGT GAT CTG AAC GGC AAC TTC ACC CTT
Asn Pro Val Leu Leu Tyr Asn Lys Met Glu Leu Gly Asp Leu Asn Ala Asn Phe Thr Leu
1201 GAG GTT GAA TCG CAG GTA TTT GAG TGG AGT TAC TTC ACA GCT AAG ATA ATG GAT ACA GTC
Glu Val Met Ser Glu Val Ser Glu Val Ser Tyr Phe Thr Ala Lys Ile Met Asp Thr Val
1261 AAC ATC AGC GTT CTT GAC ACA GAG GTC ATG AAT TAC TCC CCC AAC TAT TAC AGA AGA
Asn Ile Ser Val Pro Asp Thr Glu Lys Val Ile Asn Tyr Ser Pro Asn Tyr Tyr Arg Tyr
1321 CTC AAC CTT ATC TTG GGC AGA TAC AAC AAA AGG GAT CTG CAG CAG TAC ATG GTG TGG CGT
Leu Asn Leu Ile Leu Glu Tyr Asn Lys Arg Asp Leu Glu Asn Lys Tyr Met Val Trp Arg
1381 TTT GCT ATG AAC ATG GTG GTC GGC CTC AGC AGA TCT TAC AGG GAC ACC AGG AAA GGC TTT
Phe Ala Met Asn Met Val Val Gly Leu Ser Arg Ser Tyr Arg Asp Thr Arg Lys Ala Phe
1441 CGC AAG GCT CTG TCT GGT ACA ACG TCA GCA GCA GTC TGG CGA CAG TGT GCT CTT TAC
Arg Lys Ala Lys Ser Gly Thr Thr Ser Glu Ala Ala Val Trp Arg Glu Cys Ala Leu Tyr
1501 GTC AAC AAC AAC AAT GAC AAT GCT GTG GGA CGA CTG TAT GTG CAG GAG GGC TTC TCT GAA
Val Asn Asn Asn Met Asp Asn Ala Val Gly Arg Leu Tyr Val Glu Glu Ala Phe Ser Glu
1561 AAA AGC AAA GAA CTG ATG GAG GAG ATG ATT AAA GAC ATT CGG GAA GTG TTC ATT AGT AAC
Lys Ser Lys Glu Leu Met Glu Met Ile Lys Asp Ile Arg Asp Ile Arg Asp Thr Arg Lys Asn
1621 CTT GAT CAG CTG ACC TGG ATG GAT GCA GAG ACC AAG AAA GCA GCT GAG GAG AAG GCG GGA
Lys Asp Asp Leu Thr Trp Met Asp Ala Glu Thr Lys Lys Ala Ala Glu Glu Lys Ala Arg
1681 GCT ATT CGG GAA CGG ATC GGC TAT TCC GAG AAC ATC AAG GAT GAC AAA TAC CTT AAC AAT
Ala Ile Arg Glu Arg Ile Gly Tyr Ser Asp Asn Ile Lys Asp Asp Lys Tyr Leu Asn Asp
1741 GAG TAC AAC GAT CTG GGC TAC AGT GCA GAG GAG TAC TTT GAA AAC ATC CTA CAG AAC CTG
Glu Tyr Tyr Asn Asp Leu Ala Tyr Ser Ala Glu Glu Tyr Phe Glu Asn Ile Leu Glu Asn Leu
1801 GAG TAC GTG CAG AAG AAA CGC CTG CGG AAA CTC AGA GCT AAA GTC AAC AAA GAG GAG TGG
Glu Tyr Val Glu Lys Lys Leu Arg Lys Lys Leu Arg Val Lys Val Asn Lys Glu Glu Trp
1861 GTA ACT GGA GCT GCT GTT GTC AAC GGC TTC TAC TCA TCC AGC AAA AAT CAA ATA GTT TTT
Thr Thr Gly Ala Glu Met Val Val Asn Ala Phe Tyr Ser Ser Ser Lys Asn Glu Ile Val Phe
1921 CCT GCA GCT ATC CTG CAG CCC CTT TTC TTC AGC AAA GGC CAG GCT AAA TCT CTC AAC TAT
Pro Ala Gly Ile Leu Glu Pro Pro Phe Ser Lys Gly Glu Ala Lys Ser Leu Asn Tyr
1981 GGT GGC AAT GGC ATG GTG ATC GGT CAG GAG ATC ACA CAT GGC TTT GAT CAG AAT GGT GGT
Gly Gly Ile Gly Met Val Ile Gly His Gly Ile Thr His Gly Phe Asp Asn Asp Arg
2041 AAC TAT GAT AAA GAT GGT GAT CTG AAG GAC TGG TCG ACA CCA GGC TCC ACT GAT AGG TTC
Asn Tyr Asp Lys Asp Gly Asp Leu Lys Asp Trp Trp Thr Pro Gly Ser Thr Asp Arg Phe
2101 CTG CAG CTG TCG AAG TGC ATC GTC AAT CAG TAT GGC AAC TTT TCC TGG CAG CTG GGC AAT
Leu Asp Leu Ser Lys Cys Ile Val Asn Glu Tyr Gly Asn Phe Ser Trp Asp Leu Ala Asn
2161 GGA CTT CAT TTA AAT GGT AAC AAC CTT CTC GGG GAG AAC ATT GCT GAC AAT GGA GGG ATA
Gly Leu His Leu Asn Gly Asn Asn Thr Leu Gly Glu Asn Ile Ala Asp Asn Gly Gly Ile
2221 CGG CAG GCA TAT CAG CCC TAT AAG AAC TAT GTG GAA AAG CAT GGA GAG GCA TCA CTG
Arg Glu Ala Thr Glu Ala Tyr Lys Asn Tyr Val Glu Lys His Gly Lys Glu Pro Ser Leu
2281 CGG GGC AAT AAC CTT TCC CAG AAT CAA CTC TTC TTC CTA AAC TTT GCT CAG CTG TGG TGT
Pro Gly Ile Asn Leu Ser His Asn Glu Leu Phe Phe Leu Asn Phe Ala Glu Val Trp Cys
2341 GGA ACG CAG CGA CGA GAG CAA GCT GTT AAT TCC ATT AAA GTA GAT GTA CAG AAT CCA GGG
Gly Thr Lys Arg Pro Glu Glu Ala Val Asn Ser Ile Lys Val Asp Val His Ser Pro Gly
2401 AAA TGC AGG GTA CTG GGC TCT CTT CAG AAT TTC CAA GAA TTT GGC AAA GCA TTC AAC TGC
Lys Phe Arg Val Leu Gly Ser Leu Glu Asn Phe Pro Glu Phe Ala Lys Ala Phe Asn Cys
2461 AAA AAG AAC AGC TAC ATG GTC CTT GGC AAC ATC TCG TGT GTG TGG TGA TCC CAA GAA CTC
Lys Lys Asn Ser Tyr Met Val Pro Ala Asn Ile Cys Arg Val Trp ***
2521 TGG GAT AGT GTC GGC CTT GGC CCG CAG TAC CTC TCG CTT CTG ACT GTT GGA GCA CTC
2581 GTG GGG GGC TAC AAG GAA GTA AAG CTT CCC CTT AGC TCT ATA CTG AAT GGA CCA AGG CGG
2641 GGT GAT TGA CAC TGC AGT ATG CAG TTC CTC CCG GAA AGG CAG TGG ACT GTA CCG TCA CGG
2701 ACG AAT GGA GCA CTG TCT CTC GCA CTC TAC TAC TGT ACA TGC ACT GAT CAG GAT GAT
2761 TCC CTT ACT CAG TGG ACA CAA TCA CTC CTT CTG TTT AAT TTT TTT CAT CTT GTT TTA CTC
2821 ATC ATT TGC GAG TCT GGC CAA TGT GGT TGT GTG TGT GTG TGT GTG TGT GTG TGT
2881 GTG TGT GTG TGT GTG TGT GTG TGT GTG TGT GTG TGT GTG TGT GTG TGT GTG TGT
2941 TGT CAA GAG AGG GGC AGA CAG ACA AAT GTC CTC AAC TCC ACC GAC ATG AAT ACA CCA CCC
3001 ACC CAG CCA CCG TCA TAC ACG TGT AAC AGG CTT GAA CCA CTG TGT GTG TGT GAG GAG AAG
3061 GCT ATC CTA CCA AAC CTT TCG TTT CAG TCT ACT TCA TCT ACT TCA TCT ACT TCA TCT ACT
3121 CTA CAA TGT ATT CAA AAT GTC CTC TTT TTT AAT ACT TAG AAC TAG ATT AAG TAA ATT ATT
3181 ACA GAA AAT CTG AGT TTA TTT TCG TGA ATA CAG CAG TCG TTT ATT ATC CAG GAT AGC ATA
3241 TAC CAA AAT ACT GGT CTA TAT TTA ATA ATA ACT TAA CTC CAG TTT TCA GGC AGC TGC
3301 TCC TCA TTA GCA CTA TTT CTC GCA CTC TAC TAC TGT ACA TGC GGC TGG AAG ACA GGT GGT
3361 CAT GAA TCA TGT AGT TGA CAA TCG ACA GTC ACC AAG CCC TTT TAT ACA TTC ACT GAA CTT
3421 CCT GTG TGT TTT GTG GAT GTA TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT
3481 TTA TCT GTG GGT GTG TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT
3541 CTC TGT GTG AGC GTG TGA ATG TTT ATT TCG GGG ACG AAA AAG ACA ATG TAT CAT TGA
3601 AGC AAT AGT TTT ACT TTT TCG TGT CTT TTC TTC CCG ACT GTT ACA TTA GAA GAT CAG TAC
3661 CAC TCT GAT GTG TGT AGC GTC CTA GCA GGC GGT TGT CTT ACT TGA GCA TTA AGC TGT
3721 GAA ACA GCT AGC TTT GCT CTT CCG AAA GGT AAA AAG ACA ATG ACA TGG AAA GCT CAC TAA
3781 TGA CCT GGC TAC TTT TTT GTT TTA TAA CAA CAG TAC CAG TTT TGG TTT TGT GGA GGT
3841 TTA TGT GTC TGA CTG TTT GGT GGC TGA GAG CAG TGA CTT CTT GCA GTC TTC GGC AAG TGC
3901 CGG CCA CTT GAT GGT GAT GAG AGC TTA CAA CTT GCA CTT GCT TTT ACT CTT TGA TTT
3961 TAN TGG CTT AAA AAA AAA AAA A

Fluorescent Labeled Primer Cycle Sequencing kit, Amersham Life Science, Arlington Heights, IL, USA); (2) subcloned into pCRII and sequenced using Cy-5-labeled vector primers (Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit, Amersham Life Science); and/or (3) manually sequenced using internal primers (T7 Sequenase Version 2.0 DNA Sequencing kit, Amersham Life Science). The sequences for inserts obtained from DDPCR and library screened colonies were compared to verify that the cDNAs obtained through library screening were the same as the DDPCR fragment. Full-length sequences were compared using BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information) with known sequences in GenBank to determine similarity to known proteins.

Tissue expression

Tissue expression was determined for those DDPCR clones that showed upregulation by Northern analysis. Fish used for these studies were not primed with hCG but were obtained at the same time of the year and contained ovaries with fully mature postvitellogenic follicles. Fish were over-anesthetized and decapitated. Whole brain, heart, liver, spleen, head kidney, and gill filaments were dissected and weighed (approximately 0.2 mg each) on ice. In addition, 0.2 mg samples of the trunk kidney (opisthonephros), gastrointestinal tract (just posterior to the stomach) and muscle (anterior to the caudal peduncle) were collected. RNA/mRNA extractions and Northern blotting were performed as described above and mRNA blots were probed with full-length inserts obtained from library screening.

RESULTS AND DISCUSSION

DDPCR generates progesterin-upregulated bands in the perch ovary

DDPCR was performed on the mRNA obtained from perch ovaries following 32 h of incubation with 17,20-P (0.1 µg/ml). This particular time point was chosen since it falls just prior to the time of 17,20-P-induced ovulation *in vitro* (34–36 h) (Goetz

FIGURE 1. Complete nucleotide and amino acid sequence of PNEP-1. Nucleotides numbered on the left, amino acids on the right. The proposed methionine (ATG) start site is underlined and potential glycosylation sites are underlined twice (Asn-X-Ser/Thr). The stop codon is indicated by asterisks (**). GenBank Accession No. AF077612.

	<<<CYTOPLASMIC DOMAIN<<<L>>>MEMBRANE SP DOM<A>>>EXTRACELLULAR DOMAIN >>>	
PERCH	MPYIYIDRKFPDTSGLIQAAPAEAGDLRMETNPKSARKPRWTSLEVLGTTIVSLFIVIVALIILFATQKTDEICTTGDCQTSASRLIENMDSVDPCDNFYQYACGGWLKKNIIPE	119
MOUSE	MGRSESQMDITDINAPKPKKKQRTWPLEISLSVLVLLXTIIIAVTMIALYAT-YDDGICKSSDCIKSAARLIQNMDSVEPCTDFFKYACGGWLKKNVPIE	99
HUMAN	MKGSESQMDITDINTPKPKKKQRTWPLEISLSVLVLLTIIIAVTMIALYAT-YDDGICKSSDCIKSAARLIQNMDSATTEPCTDFFKYACGGWLKKNVPIE	99
CONCS	-----N-PK--KK-RWT-LE--L--V--L--I-L-A-T--D-IC--DC--SA-RLI-NMD---PC--F--YACGGWLK-N-IPE	
PERCH	TSSRYSTFDILRDEVLKGVLEKTDEGEATLSTRAKTLKYKCTNESLIELRGGAPLLMLPDVFEWPAVDNWNETNYGKWTWRLEDVIAKLNEKYGTQLLVNFFVGTDDRRDSNYSYIHF	239
MOUSE	TSSRYSNFDILRDEVLKDVLPQKPTEDIVAVQKAKTLYRSCINESAIDSRGGQPLLLKLPDIYGPVASDNWDQTYGTSWTAESIAQLNSKYGKKVLINFFVGTDDKNSQHIHF	219
HUMAN	TSSRYGNFDILRDEVLKDVLPQKPTEDIVAVQKAKALYRSCINESAIDSRGGQPLLLKLPDIYGPVATENWEQYKGSWTAEKALIAQLNSKYGKKVLINLFGVGTDDKNSVNHVIHF	219
CONCS	TSSRY--FDILRDEVLK-LV-----AK-LY-SC-NES-I--RGG-PLL--LPD--WPVA--NW--YG--W--E--IA-LN-KYG--L-N-FVGTDD--S---IH-	
PERCH	DQQTNLGLLSRDYYACTGYPYAEACRAYEKFMIDLAKLIRIDRGLNISETDIREVKRVMDLERDIANATDPEDRNPVLLYNKMLGDLNANFTLEVESQVFDWSYFTAKIMDTVNIISV	359
MOUSE	D-QPRLGLPSRGGYECTGIYKEACTAYVDFMISVARLIRQEQLPIDENQLSLEMNKVMELEKEIANATTKPEDRNDPMLLYNKMTLAKLQNNFSLVNGKSFWSNFTNEIMSTVNIIN	338
HUMAN	D-QPRLGLPSRDYYECTGIYKEACTAYVDFMISVARLIRQEERLPIDENQLALEMKNVMELEKEIANATKPEDRNDPMLLYNKMTLAIQNNFSLVNGKSFWSNFTNEIMSTVNIIN	338
CONCS	D-Q--LGL-SR-YY-CTG-Y-EAC-AY--FMI--A-LIR---L-I-E-----E--VM-LE--IANAT--PEDRN-P-LLYNKML-L-----NF-LE-----F-W--FT--IM-TVNI--	
PERCH	PDEKVINYSPPNYRRLNLIARYNKRLQNMVWRFAMNMVGLSRYSYDRTKAFKALSGTTSEAAVWRQCALYNNMNDNAVGRLYVQEAPESEKSKELMEEMIKDIREVFIQNLDDL	479
MOUSE	QNEEEVVVYAPYELTKLPILTKYSPRDLQNLMSWRFIMDLVSSLSRNYKESRNFALYGTSETATWRRCANYNVGNMENAVALRYVEAFAFAGESKHVVEDLIAQIREVFIQTLDDL	458
HUMAN	TNEEDVVVYAPYELTKLPILTKYSAARDLQNLMSWRFIMDLVSSLSRNYKESRNFALYGTSETATWRRCANYNVGNMENAVALRYVEAFAFAGESKHVVEDLIAQIREVFIQTLDDL	458
CONCS	---E-V-Y-P-Y---L--IL--Y--RDLQNLN-WRF-M--V--LSR-Y---R-AFRKAL-GTTSE-A-WR-CA-YVN-NM-NAVGRLYV--AF---SK---E--I--IREVFI--LDDL	
PERCH	TWMDAETKKAAEEKARAIRERIGYSDNI-KDDKYLNNNEYNLAYSAEYFENILQNLEYVQKKRLRLRVKNKEEWVTGAADVNAFYSSSKNQIVFPAGILQPPFFSKGQAKSLNYGGI	598
MOUSE	TWMDAETKKAAEEKALAIKERIGYPDDIISNENKLNNYELNLYREDEYFENIQNLKFSQSKQLKLEKVDKDEWISGAADVNAFYSSGRNQIVFPAGILQPPFFSAQSSNLNYGGI	578
HUMAN	TWMDAETKKAAEEKALAIKERIGYPDDIVSNDKLNNEYLENLYKEDEYFENIQNLKFSQSKQLKLEKVDKDEWISGAADVNAFYSSGRNQIVFPAGILQPPFFSAQSSNLNYGGI	578
CONCS	TWMDAETKK-AAEKA-AI-ERIGY-D-I-----LNNEY--L-Y---EYFENI-QNL---Q-K-L-KLR-KV-K-EW--GAADVNAFYSS--NQIVFPAGILQPPFFS--Q--SLNYGGI	
PERCH	GMVIGHEITHGFDNNGRNRYDKDGLDKDWTPGSTDRLFDLSKCIIVNQYGNFSDWDLANGHLNGNNTLGENIADNGGIRQAYQAYKNYVEKHGEEPSLPGINLSHNLFFLNFAQVWCQTH	718
MOUSE	GMVIGHEITHGFDNNGRNRYDKDGLDWDWTPGSTDRLFDLSKCIIVNQYGNFSDWDLANGHLNGNNTLGENIADNGGIRQAYQAYKNYVEKHGEEPSLPGINLSHNLFFLNFAQVWCQTH	698
HUMAN	GMVIGHEITHGFDNNGRNRYDKDGLDWDWTPGSTDRLFDLSKCIIVNQYGNFSDWDLANGHLNGNNTLGENIADNGGIRQAYQAYKNYVEKHGEEPSLPGINLSHNLFFLNFAQVWCQTH	698
CONCS	GMVIGHEITHGFDNNGRN--KDGDL-DWWT--S--F--S-C-V--YGNFSDWLA-G-HLNG-NTLGENIADNGG--QAY-AY-NY--K-GEE--LPG--L-H-QLFLNFAQVWCQTH	
PERCH	RPEQAVNSIKVDHSPGFRVLGSLQNFPEPAFAFNCKKNSYMPANICRWV	770
MOUSE	RPEYAVNSIKVDHSPGNFRITGLQNSAEFADAFHCRKNSYMPERKCRVW	750
HUMAN	RPEYAVNSIKVDHSPGNFRITGLQNSAEFSEAFHCRKNSYMPERKCRVW	750
CONCS	RPE-AVNSIK-VHSPG-FR--G-LQN--EF--AF-C-KNSYM-P--CRVW	

FIGURE 2. Amino acid alignment of the PNEP-1 protein with other known mammalian neprilysins. Amino acid sequences for perch, mouse (Chen *et al.* 1992), and human (Shipp *et al.* 1988) neprilysin were aligned using the Multiple Alignment Construction and Analysis Workbench (MACAW, National Center for Biotechnology Information). A consensus sequence (CONCS) in which all three sequences share identical residues is provided. Conserved domain structures (cytoplasmic domain (<<<CYTOPLASMIC DOMAIN<<<L>>>), membrane spanning region (T>MEMBRANE SP DOM<A>), and extracellular domain (T>>>EXTRACELLULAR DOMAIN>>>)) are indicated above the amino acid alignment. Conserved zinc-binding domains are underlined twice. Amino acids are numbered on the right and correspond to individual proteins.

et al. 1989), and is past the actinomycin-sensitive period for 17,20-P-stimulated ovulation (Theofan & Goetz 1981). For DDPCR, two anchor primers (T12MG or T12MC) were used in combination with 15 random primers (AP-1 to AP-15). This generated approximately 60 bands on DDPCR that initially appeared to be upregulated by 17,20-P. While most of these bands could be reamplified and cloned, a significant number either did not hybridize with mRNA on Northern blots or did not result in consistent upregulation with 17,20-P. In addition, based on the comparison of initial sequences for DDPCR bands, it was determined that some bands were derived from the same cDNAs. This was particularly true when multiple bands were obtained from reactions with the same primer pairs. From Northern analysis, 5 DDPCR bands were eventually verified to be consistently upregulated by 17,20-P at 32 h. Of these, one is believed to be an egg protein based on sequence homology and will be reported separately.

In all *in vitro* incubations used in this study, 17,20-P induced complete germinal vesicle breakdown by 32 h, and in the temporal Northern analysis oocytes had ovulated by the 42 h sample. In contrast, follicles in control incubates did not

undergo germinal vesicle breakdown or ovulation throughout the incubations.

Neprilysin-like perch cDNA

Using a 350 bp upregulated cDNA obtained from DDPCR, a full-length cDNA clone (PNEP-1; GenBank Accession No. AF077612) of 3985 nucleotides was obtained by library screening. PNEP-1 contains two in-frame methionine initiation sites (nucleotides 196 and 280) that would result in proteins of either 740 or 770 amino acids in length (Fig. 1). Neither of the nucleotide sequences flanking the two methionines show similarity to the Kozak consensus sequence for eukaryotic initiation sites (Kozak 1986). Assuming that the cDNA encodes for the larger 770 amino acid protein, then PNEP-1 also contains a 195 bp untranslated 5' region and a 1477 bp untranslated 3' region.

Based on amino acid similarity to GenBank sequences, the protein encoded by PNEP-1 appears to be a perch neprilysin (enkephalinase/neutral endopeptidase EC 3.4.24.11). The PNEP-1 protein is 63% identical and 74% similar to mouse neprilysin (Fig. 2). In addition to sharing a high degree of sequence similarity, the PNEP-1 protein shares

several characteristics of the neprilysin family of zinc metallopeptidases (Hooper 1994). For example, all cysteine residues are conserved in the perch neprilysin as are the two zinc binding domains that are highly characteristic of mammalian neprilysins (Fig. 2).

Neprilysin is a membrane-bound enzyme, anchored by a hydrophobic region that is flanked by a short N-terminal and long C-terminal hydrophilic stretch of amino acids (Dion *et al.* 1997). In rabbit neprilysin, the short N-terminal cytoplasmic domain consists of 27 amino acid residues; however, because the perch transcript appears to encode for a slightly larger protein, residues 1–47 may act as the cytoplasmic domain in the PNEP-1 protein. Amino acids 47–69 presumably encode for a 23 amino acid plasma membrane spanning region that is highly hydrophobic and the same length as the mammalian neprilysin plasma membrane spanning domain. Finally, amino acids 70 to 770 presumably encode for the extracellular hydrophilic domain which has 12 potential glycosylation sites (Fig. 1). In contrast, there are 5–6 glycosylation sites in mammalian neprilysins. While there are some differences between the mammalian neprilysin and the PNEP-1 protein, the high overall sequence identity and conserved neprilysin characteristics strongly suggest that the PNEP-1 protein is a perch neprilysin.

On mRNA blots of ovarian tissue probed with the original 350 bp DDPCR PNEP-1 band, a 4.0 kb transcript was significantly ($P=0.005$) upregulated by progesterin treatment at 32 h in all fish examined (Fig. 3A). Given the similar size, we assume that PNEP-1 corresponds to this transcript. The specific temporal pattern of expression of the 4.0 kb transcript was completed on the ovaries of three fish. In all three fish, levels of the 4.0 kb transcript remained low in controls but were elevated from 12 to 32 h in 17,20-P-treated ovaries (Fig. 3B). Levels of the 4.0 kb transcript decreased at 42 h; however they were still elevated in steroid incubates when compared with 42 h controls. Neprilysin has also been reported to be regulated by progestins in the human endometrium (Casey & MacDonald 1996). Specifically, in endometrial stromal cultures, neprilysin mRNA and protein levels increased in the presence of medroxyprogesterone acetate and estradiol-17 β (Casey *et al.* 1991).

Overall, four transcripts of 6.0 (results not shown), 4.0, 2.5, and 1.0 kb were detected when PNEP-1 was used to probe mRNA Northern blots of other perch tissues (Fig. 3C). The larger 6.0 kb transcript was detected in the heart and trunk kidney (data not shown), while the 4.0 kb transcript that was regulated by 17,20-P in the ovary, was also present in high abundance in the heart. A smaller

FIGURE 3. Northern blots of PNEP transcripts in the ovary (A and B) and in other perch tissues (C). Each lane represents 0.5 μ g mRNA. (A) Expression of PNEP mRNA at 32 h under 17,20-P stimulation (0.1 μ g/ml, S) or ethanol vehicle (1 μ l/ml, C) in the ovaries from five fish (F1–F5). The 4.0 kb transcript was upregulated by 17,20-P in all five fish assayed ($P=0.005$; pair-wise *t*-test). (B) Representative Northern blot of the temporal expression pattern of PNEP ovarian transcripts in one fish. Numbers above the lanes represent hours of incubation in Cortland medium with 17,20-P (0.1 μ g/ml, 17,20P) or ethanol vehicle (1 μ l/ml, Control). Two other fish assayed showed a similar pattern of expression. (C) PNEP transcripts in other perch tissues. H. Kidney, head kidney; Ovary-C, ovary at 42 h in the presence of ethanol vehicle; Ovary-S, ovary at 42 h under 17,20-P stimulation.

band encoding for a 2.5 kb transcript was detected in the gills, gut, heart, head kidney, liver, spleen, and ovary. Finally, a small 1.0 kb transcript was

1 CCG CAC GAG CCA GGG AGT GAC AGA AAA AGT AGG AGG GAG GAA GAG TGG AAT AGA GAG
61 CAG GAG CAA AGC CAC AGC CTT CTT TTC CTT CAG TCC TCT CTT CTT TCC TCC TCC TCC TCC TCC
121 CTT CAG CCG GAG AGC AGC AGC AGT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
Met Leu Ser Leu Gln Ser Leu Ser Thr Leu Leu Leu Leu 13

181 CTT CTT CTT TAC CCG CTT CCG CTT CCG CAG GAG CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
Leu Leu Leu Tyr Pro Pro Pro Val Ser Ala Gln Gln Val Leu Val Arg Leu Ala Gln Val 33

241 GCT GCT CCG AAT GCG AGC GAG CAG CTT GAG CAG CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
Gly Val Arg Asn Ala Asn Gln Gln Arg Val Gln Val Phe His Asn Gln Ala Thr Gln Gln Thr 53

301 GTT GTT GAG CAG GAG GTG GAT CTT AAT AGT GCG AAT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
Val Cys Asp Asp Gln Val Asp Leu Asn Met Ala Asn Val Val Cys Arg Gln Leu Gln Gln Thr 73

361 CAA CCG GCG TTG ACC TGG CCA CAG AGT GCG AAG TTT GCG GCG GAG CAG CTT CTT CTT CTT CTT
Gln Arg Gln Leu Thr Thr Ala His Ser Ala Lys Phe Gln Gln Gln Gln Gln Leu Thr 93

421 TTG GAG AAT GTG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Leu Asp Asn Val Arg Cys Thr Thr Gln Leu Ser Leu Thr Ala Asn Cys Arg Ser Ser Asn Gln 113

481 TGG GAG AAT GTG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Trp Gln Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 133

541 AGA GCG GCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Arg Pro Gln Ser Pro Ala Val Ala Val Gln Gln Ala Pro Ser Ser Ser Ser Ser Ser Ser Ser 153

601 AAC CAG CCA GAA CAG AGA AAC CCA CCA CCG CTT CCA CAG TTT GTG CCA CCG CCG CCG CCG CCG
Asn Gln Pro Gln Gln Arg Asn Pro Pro Pro Leu Pro Gln Ser Val Pro Pro Pro Ala His 173

661 ATC TCG TCA TTT TCT CCG AGA GCG CAG AGT AGT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
Ile Ser Ser Ser Ser Ser Ala Arg Gln His Gln Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 193

721 CTT CCG AAT GTG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Arg Arg Ser Ile Thr Ser Pro Gln Gln Asn Gln His Gln Thr Thr Thr Thr Thr Thr Thr Thr 213

781 CCA GCT AAT GTG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Arg Gln Ser Arg Arg Ala Ser Pro Gln Val Asn Pro Ala Leu Pro Gln Gln Gln His Gln Leu 233

841 CTT TCA CTT GTG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Leu Ser Arg Leu Ala Asn Gln Tyr Arg Gln Ser Gln Asp Thr Ala Val Arg Arg Gln Thr 253

901 GAG GAG CCG AAG AGC CAG TTT CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Gln Gln Arg Thr Asn Arg Gln Phe Gln Pro Gln Ser Gln Arg Arg Ser Arg Arg Arg Arg Arg 273

961 GAG CTT AAT GTG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Gln Leu Ser Gln Asn His Val Gln Pro Asp Leu Gln Leu Thr Thr Thr Thr Thr Thr Thr Thr 293

1021 CAG GAG TCT GAT AGT CCG TTA GAG GCG CTT CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Gln Gln Ser Asp Arg Val His Leu Gln Gln Ala Arg Leu Arg Pro Val Leu Ser Ser Asn 313

1081 CAG GCG CTT GTG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
His Gln Gln Gln Val Met Gln Gln Val Val Leu Gln Val Lys His Ala Gln Arg Thr Arg His 333

1141 GTT GTT AAG CAG GCG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Val Cys Asn Gln Gln Gln Thr Asp Asn Leu Ser Ser Ser Arg Val Val Cys Gln Met Leu Gln Phe 353

1201 CCG GCG TCG TCG TTT CAG CCA AAC GCG TAC AGG AAA CCG CCG CCG CCG CCG CCG CCG CCG CCG
Pro Ala Ala Gln Val Phe Asn Gln Asn Ala Tyr Arg Lys Leu Thr Asp Asn Ser Lys Leu Ala 373

1261 GAT CTT CCG TCG TCG TTT CAG CCA AAC GCG TAC AGG AAA CCG CCG CCG CCG CCG CCG CCG CCG
Asp Pro Ser Ser Arg Leu Arg Thr Gln Thr Ser Lys Lys Leu Ala Tyr Trp Val Gln Lys Val 393

1321 CAG TTT CAG CAG CAG GAG GTT CTT GTG TCA CAG TCC CAG GCG CCG CCG CCG CCG CCG CCG CCG
Gln Cys Gln Gln Arg Gln Val Ser Leu Ser Gln Cys Gln Ala Gln Leu Ser Leu Pro Arg 413

1381 GAT GAT GCT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Ser Asp Val Pro Cys Arg Asp Gln Met His Ala Val Val Arg Cys Val Pro Gln Ser Gln 433

1441 TTT CCA CTT TAT CCG AGA CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Phe Thr Arg Tyr Gln Arg Ala Pro Pro Ala Val Thr Pro Val Thr Pro Val Thr Arg Lys 453

1501 GCG GCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Gly Gln Pro Cys Leu Gln Gln Gln Arg Val Gln Val Gln Leu Gln Gln Gln Gln Gln Gln Gln 473

1561 GTT GTT GAG CAG CAG TGG AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC AGC
Val Cys Asp His Leu Thr Met Met Ala Ala Ser Val Val Cys Arg Gln Leu Gln Gln Thr 493

1621 AGC AGC CCG AAA GAG CCG CTT ACC AAA GCT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Gly Thr Ala Lys Gln Ala Leu Thr Lys Ala His Leu Gln Gln Gln Gln Gln Gln Gln Gln Gln 513

1681 ATG AAC AGC TCG TCG TTT CAG CCA AAC GCG TAC AGG AAA CCG CCG CCG CCG CCG CCG CCG CCG
Met Asn Ser Val Gln Cys Gln Tyr Arg Gln Lys Ser Thr Thr Gln Gln Thr Thr Thr Thr Thr 533

1741 GTT CCG CTT TAC CAG TCC AAA CAG CAG GAT GTG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Val Pro Leu Tyr Ser Cys Lys His Asn Gln Asp Val Ala Val Arg Cys Asn Val Pro Asn 553

1801 ACT GGT AGT CAG CAG TTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Thr Gln Met Gln Ala Thr Val Arg Leu Ala Gln Gln Arg Gln Pro Ser Gln Gln Arg Val 573

1861 GAG GTT CAG CAG CAG TTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG
Gln Val Leu Met Gln Val Gln Gln Val Arg Val Arg Trp Gln Ser Thr Cys Ser Gln Asn Thr 593

1921 GGC ATC AAC GCG CCG AGT GTC GTC TTT CCA CAG CTT GGT TTT GGT TTT GGT TTT GGT TTT GGT
Gly Ile Asn Gln Ala Met Val Val Cys Arg Gln Leu Gln Leu Gln Gln Phe Ala Ser Ser Ala 613

1981 CAG CAG AGC TGG TAC TGG CCG GGT TTT TCA GAT GCG CCG CCG CCG CCG CCG CCG CCG CCG
His Gln Gln Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 633

2041 ACC CAG TTT AGC ACT GAG AGT TCT TCT CAG CAG TCC CCG AGA AAC ACA AAT GTT TAC
Thr His Cys Ile Gln Thr Gln Met Ser Thr Gln Gln Cys Arg Arg Asn Thr Asn Val Thr 653

2101 TTT CCG AGA GAG GAT GCG AGA CTT CCA GAG GTT ACA TTT GTG AGA CTT CCG CCG CCG CCG
Cys Pro Arg Gln Gln Gln Arg Ala Ala Val Thr Thr Cys Val Gln Thr Ala Pro Asp 673

2161 CTT CTT CTT GAT CTT CAG CTT CTT CCG AGC AGC CCG TAC CTT GAG GAG CCA CCG CCG CCG CCG
Leu Val Leu Asp Ala Gln Leu Val Gln Gln Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 693

2221 CTT CTT TCC TCC CTT AAT GAG GAG AAC TCC CTT TCC TCC TCC CCG AGG AGT AAC TCC
Leu Leu Ser Cys Ala Asn Gln Gln Asn Cys Leu Ser Ser Ser Ala Ala Thr Thr Thr Thr Thr 713

2281 CCG TCC GAG CAG CCG CCG CTT CTT CTT TCC TCC TCC CCG ATC ATG AAC AGT GCG CCG CCG
Pro Tyr Gln His Arg Arg Leu Leu Arg Phe Ser Ser Arg Ala Met Asn Met Gln Arg Ala 733

2341 GAC TTT CCA CCG CCA GCG AGC AGA GAA AGT TGG ACA TGG CAG CAG TCC CAG AGG CAG TAC
Asp Phe Arg Arg Arg Ala Thr Arg Gln Ser Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 753

2401 CAG AGC AAT GAG GTT TTT ACC CAG TAC CAG CTT CTT CCG CTT CCG CCG CCG CCG CCG CCG
His Ser Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 773

2461 GAG GGT CAG AGC AGC AGC TTT TTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT CTT
Gln Gln His Lys Ala Ser Phe Cys Leu Gln Asp Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 793

2521 CCG TTT CTT TAT TAT TAT CAG CAG GAG GGT ATT TCA GTT GAT TTT GAT TTT GAT TTT GAT
Arg Tyr Ala Cys Tyr Asn Met Gln Gln Gln Gln Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 813

2581 CCG CAG GAT ATT GAG TCC CAG TGG GTT CCG ATC ACA GAG ACA CCG CCG CCG CCG CCG CCG
His Asp Ile Asp Cys Gln Thr Val Asp Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr 833

2641 TTT CAG GTT GAG GTT ACC CTT TTA GAG AGT GCG AGC AGC AGC AGC AGC AGC AGC AGC AGC
Phe Gln Val Gln Val Asn Pro Thr Leu Asp Met Ala Gln Thr Thr Thr Thr Thr Thr Thr Thr 853

2701 ATG CCG TTT CCG TAT TAT TAT GAT GAG GAA CCA GTT TAC AGT TTT GAG TCC CAG TCC CAG
Met Arg Cys Arg Cys Lys Tyr Asp Gln Gln Arg Val Thr Thr Thr Thr Thr Thr Thr Thr Thr 873

2761 GAT CTT TAC ACC GCG GTG GAG GAG TTT TTT GAG CAG CAG CAG CAG CAG CAG CAG CAG CAG
Asp Ala Tyr Ser Ala Gln Val Gln Asp Leu Phe Asp His Gln Arg Gln Thr Thr Thr Thr Thr 893

2821 TTT CTT TGA ATT AAG GAA CCA GAT TTA AGG ACA GAA CCA CTT TAC AAC TTT TCA CCA
Phe Leu *** 895

2881 CAC CTT AAC AGT TCA GGG GAA GAA GGT TAA ATG ACA ATG ACA TCC CAG TGG ATG GAA AGA
2941 AGA GGG TCA GAG GAA GAA GGG TGG GAA ACT GGG ATG TGG TGG GAG CTT CAT GCG AAA
3001 TGA TTA ATT AGT AGT CCG CCA CTT GAC AGA AAA AGA CTA AGT TAA ACT ATC ACA AGT ATC
3061 ATG TCA CCA TAA TAA AAC AGA GAG TGG CTT CCA ACT TTT TTA AAA GTG TTT CTT TTT TTT
3121 AAC AAA TTT AAT TTT CTT GAT CAA AAC TTA ACT ACA GTT AAG AAA GTG ATA AGA TAA AGG
3181 CAC AAT GAG ACC AAA CCA TCC TTT ACT CTT ACA GCG CAG CAG CAG CAG CAG CAG CAG CAG
3241 AAA TCA ACT CAG TGA ACA GAG TTT CTA GAA AAC TTT CTA CAG CTT TTT CTT TTT TTT
3301 GTT GAG ATT ACT AAT CCA GTT TTT ACT GTT ACA ACT GTG TGG TTT GTT TTT TTT TTT
3361 ATT GTT TTT TTT CAG CAG CTT CTT ACT GTT CTT TTT GAA TAA ACA TTT TAA TTT ATT GCA
3421 AAA AAA AAA AAA AAA

detected in all tissues assayed with the exception of the brain. Multiple neprilysin transcripts ranging from 2·7 to 5·7 kb have also been reported in various mammalian cells or tissues (Shipp *et al.* 1988, Chen *et al.* 1992). In addition, localization of perch neprilysin mRNAs in various tissues throughout the body is consistent with the previous observation that neprilysin is located in the kidney, lung, intestine, and parts of the central nervous system in mammals (Erdos & Skidgel 1989).

Mammalian neprilysin inactivates small peptides such as enkephalins, substance P, atrial natriuretic peptide, endothelins, bradykinin, neurotensin, and chemotactic peptide (for review see Erdos & Skidgel 1989). Some of these factors have been observed in the vertebrate ovary (Ackland *et al.* 1992) and some have also been associated with the ovulatory process (Murdoch & McCormick 1989, Hellberg *et al.* 1991, Furudate *et al.* 1994). Given that many of the small peptides which are cleaved by neprilysin are vasoactive, one possible role for neprilysin in the ovary would be to regulate the activity of these peptides and, as a consequence, influence follicular blood flow. Alternatively, peptides such as endothelins have also been associated with the control of ovarian steroidogenesis (Tedeschi *et al.* 1992), and kinins have been implicated in oocyte expulsion (Hellberg *et al.* 1991). Thus, the actions of neprilysin in the ovary may extend beyond vascular control. Further, since neprilysin can cleave chemotactic peptides (Painter *et al.* 1988), it may regulate the migration of leukocytes that is observed in the ovary at the time of ovulation (Brannstrom *et al.* 1993).

Lysyl oxidase-like perch cDNA

Using a 257 bp upregulated cDNA obtained from DDPCR, several larger cDNAs were eventually obtained by library screening and fully sequenced. The largest cDNA was 3438 bp (PLO-2; GenBank Accession No. AF143003) and contained an open reading frame that was 2685 bp, presumably encoding a protein of 895 amino acids (Fig. 4). A slightly smaller cDNA (3354 bp; PLO-1, GenBank Accession No. AF103901) was also completely sequenced (results not shown). PLO-1 encoded for a protein that was nearly identical with PLO-2, but did not contain an initiation codon and lacked the N-terminal 73 amino acids of PLO-2. In addition,

FIGURE 4. Complete nucleotide and amino acid sequence of PLO-2. Nucleotides numbered on the left, amino acids on the right. The stop codon is indicated by asterisks (***). GenBank Accession No. AF143003.

FIGURE 5. Amino acid alignment of the PLO-2 protein with other human lysyl oxidase proteins. Amino acid sequences for perch (PLO2), human lysyl oxidase-related protein (LOR) (Saito *et al.* 1997), human lysyl oxidase (LO) (Hamalainen *et al.* 1991) and human lysyl oxidase-like protein (LOL) (Kenyon *et al.* 1993) were aligned using MACAW. A consensus sequence (Con) in which perch and human LOR protein share identical residues is provided. Similarly, asterisks (*) below the consensus sequence denote identical (Id) residues in all four proteins. Amino acids are numbered on the right and correspond to individual proteins.

the untranslated 5' region of PLO-2 was also missing. In contrast, PLO-1 contained 273 bp in the 3' untranslated region that were not found in PLO-2. Even though PLO-2 appears to contain a complete open reading frame, it is probable that this is still not the complete cDNA since a single ovarian transcript of just less than 4.0 kb is detected on Northern blots using these cDNAs as a probe (see below).

The presumed protein encoded by the PLOs is similar to the lysyl oxidase enzyme family that includes lysyl oxidase (LO) (Hamalainen *et al.* 1991), lysyl oxidase-like protein (LOL) (Kenyon *et al.* 1993), and lysyl oxidase-related protein (LOR) (Saito *et al.* 1997). These proteins show the greatest similarity to one another and to the PLO protein in the carboxy region (Fig. 5). Overall, LO, LOL, LOR and PLO proteins are 41.2% identical over a C-terminal region of 206 amino acids. However, of the three mammalian lysyl oxidase proteins, the perch protein is most similar to the LOR protein in

the same C terminal region (68.0% identity). Over the entire LOR sequence, PLO-2 encodes for a protein that is 48.4% identical to LOR. A direct comparison of all of the lysyl oxidase proteins is complicated by the fact that LOR and the PLO proteins are significantly longer than LO or LOL, and the alignment suggests that both LOR and the PLO proteins are greatly extended between the extreme amino and carboxy regions (Fig. 5). In fact, the PLO-2 protein is the longest lysyl oxidase-like protein yet characterized.

The PLO cDNAs detected a single transcript on Northern blots of perch ovaries that was slightly less than 4.0 kb. This transcript increased significantly ($P=0.01$) at 32 h in all ovaries treated with 17,20-P (Fig. 6A). In temporal experiments on the ovaries of three fish, PLO transcripts increased in the ovary at 32 and 42 h following 17,20-P stimulation while PLO transcripts were not detected in any controls or in 17,20-P-treated ovaries at 12 or 24 h (Fig. 6B). A glucocorticoid

FIGURE 6. Northern blots of PLO transcripts in the ovary (A and B) and in other perch tissues (C). Each lane represents 0.5 μ g mRNA. (A) Expression of PLO mRNA at 32 h under 17,20-P (0.1 μ g/ml, S) or ethanol vehicle (1 μ l/ml, C) in the ovaries from seven fish (F1-F7). The 4.0 kb transcript was upregulated by 17,20-P in all seven fish assayed ($P=0.01$; pair-wise t -test). Note: longer exposure time is required to observe upregulation in fish F5. (B) Representative Northern blot of the temporal expression pattern of PLO ovarian transcripts in one fish. Numbers above the lanes represent hours of incubation in Cortland medium with 17,20-P (0.1 μ g/ml, 17,20P) or ethanol vehicle (1 μ l/ml, Control). Two other fish assayed showed a similar pattern of expression. (C) PLO transcripts in other perch tissues. Kidney, trunk (opisthonephros) kidney; all other abbreviations as in Fig. 3.

response element is present in the 5' flanking region of the mammalian LOL gene (Smith-Mungo & Kagan 1998), thus supporting the hypothesis that steroids can directly regulate the expression of these proteins.

PLO transcripts appear to be present only in the 17,20-P-stimulated ovary; however a very low expression of what appears to be a slightly smaller transcript may occur in the brain and gills (Fig. 6C). Human LO and LOL are expressed constitutively

in high abundance in many different tissues. Specifically, LO has been localized to human heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas while LOL transcripts have been detected in human lung, kidney, placenta, liver, brain, pancreas, heart, and skeletal muscle (Kim *et al.* 1995). Although the tissue localization of human LOR mRNA and protein have not been studied, LOR transcripts were first detected in fibroblast cells and are constitutively expressed in fetal lung fibroblast cell lines (Saito *et al.* 1997). Since the 4.0 kb perch PLO transcript was detected exclusively in the ovary, this may indicate that it is either nonconstitutively expressed and induced only when needed by various mediators, or is a novel ovarian specific lysyl oxidase gene upregulated at ovulation and/or inflammation.

The final step in the posttranslational modification of collagen within the extracellular matrix is the crosslinking of collagen chains and fibrils (Franklin 1997). Lysyl oxidase is the enzyme that deaminates lysine residues causing spontaneous crosslinking between collagen fibrils, thus making the collagen tough and insoluble. The same enzyme also acts on elastin, producing similar cross-linking. Based on their similar protein structures, the other members of the lysyl oxidase family of proteins are also believed to be able to cross-link collagen and elastin fibers (Kim *et al.* 1995, Saito *et al.* 1997). Since ovulation is marked by an increase in protease activity and follicle degradation (Espey 1980, Goetz *et al.* 1991), restructuring of the ovary following ovulation would be expected. Lysyl oxidase activity could be involved in tissue repair following ovulation by crosslinking collagen or elastin fibrils. In the perfused rabbit ovary, lysyl oxidase activity peaks just after ovulation and was hypothesized to play an integral role in gonadal restructuring (Himeno 1986). However, in that study lysyl oxidase activity was assayed indirectly, and LO protein or mRNA characterization was not conducted (Himeno 1986). Given the structure of the perch lysyl oxidase protein isolated in the present study and the apparent ovarian-specific expression, an intriguing hypothesis is that the lysyl oxidase enzyme activity observed in rabbits after ovulation actually results from other lysyl oxidase enzymes instead of LO itself. Regardless, upregulation of PLO transcripts by 17,20-P at 42 h (postovulation), may indicate that lysyl oxidase enzyme activity regulates gonadal restructuring in perch in a similar fashion as hypothesized for rabbits.

As is the case for neprilysin, lysyl oxidase proteins have been implicated in the stimulation of other physiological responses in addition to the cross-linking of collagen and elastin. For example, LO

1 AAT TCG GCA CGA GAG CAG ACC AAC CTT CCT AAC TCA GAC ATG GCT GAC CAA CTA ACA GAA 7
Met: Ala Asp Gln Leu Thr Glu
61 GAG CAG ATT GCA GAG TTC AAG GAG GGC TTC TCC CTG TTT GAT AAA GAG GCA GAT GGC ACC 27
Glu Gln Ile Ala Phe Lys Glu Ala Phe Ser Leu Phe Asp Lys Asp Gly Thr
121 ATC ACC ACT AAG GAG CTG GGC ACT GTC ATG AGG TCA CTG GGT CAA AAC CCA ACT GAG GGC 47
Ile Thr Thr Lys Glu Leu Thr Val Met Arg Ser Leu Gly Gln Asn Pro Thr Glu Ala
181 GAG CTC CAG GAC ATG ACT AAT GAG GTG GAC GCA GAT GGC AAC GGG ACA ATT GAC TTC CCT 67
Glu Leu Gln Asp Met Ile Asn Glu Val Asp Ala Asp Gly Asn Gly Thr Ile Asp Phe Pro
241 GAG TTC CTG ACA ATG ATG GCG AGG AAG ATG AAG GAC ACT GAT AOC GAG GAG GAG ATC AOG 87
Glu Phe Leu Thr Met Met Ala Arg Lys Met Lys Asp Thr Asp Ser Glu Glu Glu Ile Arg
301 GAG GCA TTC AGG GTC TTT GAT AAG GAT GGC AAC GGT TAC ATT AOC GCA GCA GAG CTG CGT 107
Glu Ala Phe Arg Val Phe Asp Lys Asp Gly Asn Gly Tyr Ile Ser Ala Ala Glu Leu Arg
361 CAT GTG ATG ACT AAC CTG GGG GAA AAG CTG ACC GAT GAG GAA GTA GAG GAG ATG ATC AGA 127
His Val Met Thr Asn Leu Glu Lys Leu Thr Asp Glu Glu Val Asp Glu Met Ile Arg
421 GAA GCA GAC ATC GAT GGA GAG GAG GTC AAC TAT GAA GAA TTT GTT CAG ATG ATG ACC 147
Glu Ala Asp Ile Asp Gly Asp Gly Gln Val Asn Tyr Glu Glu Phe Val Gln Met Met Thr
481 GGC AAA TGA ACT GGC TTC ACA TTA AAG ACA AAG AAT CAG TCA AAT GTT TCA CTT ACC TCT 149
Ala Lys ***
541 TAT TCG AAA AAT CTA CAT TTA TTC ATA CTG TGT TGT ATA GAC AAC TTA AAC TGA ATG TTG
601 GAA AAC TGA AAA TCT GTC CAT ATA AAC AAG CTC AAA AAA GGT AAA AAG ACA AAA CGG AAA
661 ATT CTA AAT GAA TCT GCA TGT ACT GGC TTG TAT TCC CCG CCC CCC AGC GCT GAG CTG CCC
721 AAT CAG ATC CTA CTC TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG TCG
781 TCC TCT GCT TCC TCG TCC CAT CTG CTT GAT GTC CCG ATG ATC ATA TCG GCT GTT CAA TCG
841 CCG TTC TTC TAT GAC TTC CTG TTT TTC TTT TTC TTT TTC TTT TTC TTT TTC TTT TTC TTT
901 TTA GAT GGG TTA CTC TTA GAA GGC GAA TGG CAG AAG GCT TTT TGG CCG CAT GGT GTT CTG
961 GCA CCC ACA TGG AGG AGC GAG GGC TTA GTT GAA AGA TGG ATG GGA GTG AGT GAA CCA TCG
1021 GGA CTT TAG CCG TTT GCT TCC CTT AAT GAA GTC AGT GAT AAT AGA TAT TTT TAA GAA ATT
1081 TTT TAA AAT GGA ATA AAT AAG AAC TCA TTT TAT ATT CTG TGT TTA TCG CAT GTC AGG ATA
1141 CTG AAT TGA TCC TTT GTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT
1201 AGC CCC TTT GGT GTT GGA TTT GTA TCA GAC TTA TAA GAG GGG TTT GGT GGG CTT CAA AGT
1261 TGA TGA TAT ACA AGA GGC TCT TTT AGG CAA TAT CCA TAT CAT TAA TAT CAG GTT TTG CAA
1321 ACT TTG GAG GTT TAC AAA GCA TGA ATC TGT GAT TAT CAC TGA AGC ATG TTT CCG TAC TGT
1381 ATA TTG CCT CAA AGG TTC CAT GTG TCT ACG GGT GGG GGG GGG GGT GAT TTC AGT AGT GGG
1441 GAC AGG GTC GGT GCT TCG GGG CAT TTC TAT CTT GTG TTT GTG TGT GAG TAT GCG TCG GTC
1501 TTT GGG TCG GCG GAG GCG CCG GGT CCA ACT GTT CTG TCG AGT TCA GTT GAT GTG CAT
1561 TCC AAG TTG TAC AAG CTA GTC TTT ACA TTT TGT TTT TCC AAT AAA ATA CCA CCA ACT AAC
1621 ATC TCG AAA AAA AAA AAA AAA AAA

FIGURE 7. Complete nucleotide and amino acid sequence of perch calmodulin. Nucleotides numbered on the left, amino acids on the right. The proposed methionine start site is underlined (Met). The stop codon is indicated by asterisks (***). GenBank Accession No. AF085250.

acts as a monocyte chemotactic substance (Lazarus *et al.* 1994) and thus could be involved in leukocyte migration into the ovary at ovulation. Further, the inherent enzyme activity of LO produces hydrogen peroxide as a byproduct (Green *et al.* 1995), and it has been shown in fish that low levels of hydrogen peroxide can induce ovulation *in vitro* when combined with certain signal transduction mediators such as sodium orthovanadate (Hsu & Goetz 1991).

Yellow perch calmodulin

Using a 190 bp upregulated cDNA obtained from DDPCR, a 1644 bp clone (PCAL-1; GenBank Accession No. AF085250) was obtained by library screening. PCAL-1 contains a 447 bp open reading frame that is initiated at a methionine encoded by nucleotides 40–42 (Fig. 7). The cDNA also contains a 39 bp 5' untranslated region and a 1104 bp 3' untranslated region.

Based on sequence comparison with GenBank, the protein encoded by PCAL-1 is definitely calmodulin. At the amino acid level, the open reading frame of this clone is 100% identical to other vertebrate calmodulins including those from *Xenopus laevis* (Chien & Dawid 1984), medaka (Matso *et al.* 1992), humans (Fischer *et al.* 1988), and chickens (Putkey *et al.* 1983). At the nucleotide level, PCAL-1 is approximately 86% identical to other vertebrate calmodulins.

FIGURE 8. Northern blots of calmodulin transcripts in the ovary (A and B) and in other perch tissues (C). Each lane represents 0.5 µg mRNA. (A) Expression of perch calmodulin mRNA at 32 h under 17,20-P (0.1 µg/ml, S) or ethanol vehicle (1 µl/ml, C) in the ovaries from seven fish (F1-F7). The 1.6 kb transcript was upregulated by 17,20-P in all seven fish assayed ($P=0.01$; pair-wise *t*-test). (B) Representative Northern blot of the temporal expression pattern of calmodulin ovarian transcripts in one fish. Numbers above the lanes represent hours of incubation in Cortland medium with 17,20-P (0.1 µg/ml, 17,20P) or ethanol vehicle (1 µl/ml, Control). Of three other fish assayed, two showed a similar pattern of expression and one did not. (C) Calmodulin transcripts in other perch tissues. Abbreviations as in Figs 3 and 6.

Two transcripts (1.6 and 0.6 kb) were detected on Northern blots of ovarian RNA when probed with PCAL-1; however, only the larger 1.6 kb transcript was significantly ($P=0.01$) upregulated by 17,20-P after 32 h of incubation (Fig. 8A). Temporal mRNA Northern analysis was completed on the ovaries of four fish and confirmed that only the 1.6 kb calmodulin transcript was regulated by 17,20-P. Three of the four fish assayed showed strong upregulation of calmodulin transcripts at 32 and/or 42 h (Fig. 8B). One fish exhibited a variable

1 GGC ACG AGC AGC TAC AAG ATC GGG AAC CAC TCT AGT ACC GTC AAT GTT AGT GGG TCT GGA
 61 CAT GTT GGA AGC ATT TAT ATT AGC GGC GCA GGT TCC ACT GTG ATT GAA CCA TGG AGC
 His Val Gly Thr Ile Tyr Met Arg Ala Ala Arg Ser Phe Thr Val Ser Glu Pro Trp Arg
 121 AAA ATA TCT TGG GAC AAT GCG AGG GAT CTC CAG TAT GTG AAG GAG TAT AAG OCT GGA
 Lys Ile Ser Trp Gly Asp Asn Ala Arg Asp Leu Gln Gly Arg Val Lys Glu Tyr Lys Pro Gly
 181 AAC AAG GAC ATC AAA CAT GTC CGA ATC CTT CTT TAT GGA CCT GAC GGA GCT GGA AAG TCC
 Asn Lys Asp Ile Lys His Val Arg Ile Leu Leu Tyr Gly Pro Asp Gly Ala Gly Lys Ser
 241 AGC TTC ATC AAG TCT GTC TGC AGC GTC ATA CAA GGC AGA GTG ACT ACT GAT GCT TTG ACC
 Ser Phe Ile Lys Ser Val Cys Ser Val Ile Gln Gly Arg Val Thr Thr Asp Ala Leu Thr
 301 TCT GAC AAA AGT TAC AAT GGA ACA TAT GAA AGC TTT AAA ATC AGA AAA GGA AAC TCA
 Ser Asp Lys Ser Tyr Asn Gly Thr Tyr Glu Thr Phe Lys Ile Arg Lys Glu Gly Asn Ser
 361 GAG ACA TTC TGT CAT TTC GTC TTC AAT CAC GTC ATC GAT CAG AAG GAT GGA GAT GGA ATC
 Glu Ser Phe Cys His Phe Val Phe Asn Asp Val Met Asp Leu Lys Asp Gly Asp Gly Ile
 421 TGT GCT GAC GAC ATC AAA CTG GCC CTG AAA GGA CAT GTG AAG GAG GGT TAC CAG TTC AAG
 Cys Ala Asp Asp Ile Lys Leu Ala Leu Lys Gly His Val Lys Glu Gly Tyr Gln Phe Lys
 481 CCT TCA TCT CCA CTG TCT CTT GAG GAT CCA GGC TAC AAC CCC COT CCC TCT GCA GAC GAC
 Pro Ser Ser Pro Leu Ser Pro Glu Asp Pro Gly Tyr Asn Pro Arg Pro Ser Ala Asp Asp
 541 AAA GTC CAT GTT GTC GTT TGT GTG CTT TCT GCT GAC TCA GGA GGA ATT ACA GAC TCA GTT
 Lys Val His Val Leu Val Cys Val Leu Ser Ala Asp Ser Ala Gly Ile Thr Asp Ser Val
 601 CTG AAG AAG ATG GGC GAC ATC AGA GAG GCA GCC AGT GAG CTG GCG ATT CCC CAA ATG GCA
 Leu Lys Lys Met Ala Asp Ile Arg Glu Ala Ala Ser Glu Leu Gly Ile Pro Gln Met Ala
 661 ATA GGC ACC CAC ATT GAT TCA GCC TGT CTT GAA ACT AAA AAG GAT CTG AAG AAT GTG TAC
 Ile Gly Thr His Ile Asp Ser Ala Cys Pro Glu Thr Lys Lys Asp Leu Lys Asn Val Tyr
 721 AAG AGC CAG CAG CTG AAG CAA AAG GTG AAA GAT TCT AGC GCA GCA GTG GGA GTC CCA GAG
 Lys Ser Gln Lys Lys Gln Lys Val Lys Asp Phe Ser Ala Ala Val Gly Val Pro Glu
 781 AAC TAC ATC TTT CTT GTC AAG AAC TAC AGT GAT GAA ACC CAC ATC AAT GAT GAT GTC AAC
 Asn Tyr Ile Phe Pro Val Lys Asn Tyr Ser Asp Glu Thr Thr His Ile Asn Asp Asp Val Asn
 841 ACT CTG ATC CTG AGT GCA CTG AGA CAA ATG ATC AGC CTT GGA GAC AAC TTC ATT GAG AAA
 Thr Leu Ile Leu Ser Ala Leu Arg Gln Met Ile Ser Leu Gly Asp Asn Phe Ile Glu Lys
 901 TGA ATC TAA ATA TTA AAA GAG AGT CTG GGA TGT CCA GTT ATA ATC TGT TGG CAG ATA

 961 GCT TTG ACA GTT ATT GTC TGT ATT TGA TGA AGA CTG TTG TCT TCA TAA GAC AGC TGG GAT
 1021 CTG ATC CTA GTT ACC ACA TAC ATC TAA TTT CAT GTT TGC ATG TTT GTG ATG AAT AAA
 1081 TTC TGT GGC TGG GCA AAA AAA AAA AAA A A

FIGURE 9. Complete nucleotide and amino acid sequence of PMAP-1. Nucleotides numbered on the left, amino acids on the right. The proposed methionine start site is underlined (Met). Potential glycosylation sites are underlined twice (Asn-X-Ser/Thr). The stop codon is indicated by asterisks (***). GenBank Accession No. AF085251.

pattern of transcript expression in which no difference between steroid treatment and controls was observed at most time points (data not shown). However, calmodulin transcripts in this fish increased in 17,20-P-stimulated incubates as time progressed, indicating that calmodulin upregulation by 17,20-P occurred in this fish as well.

The large 1.6 kb calmodulin transcript was detected in all other tissues assayed (Fig. 8C).

Calmodulin is found throughout the body and has been detected in all living cells to date (Celio *et al.* 1996). Thus, the ubiquitous expression of calmodulin in perch is not surprising.

Calmodulin mediates many cellular responses including cell signaling, gene regulation, DNA synthesis, mitosis, smooth muscle contraction, and apoptotic cellular events (Celio *et al.* 1996). Thus, delineating a specific role for calmodulin in the ovary may be difficult. However, it appears that calmodulin plays a role in inducing ovulation in rabbits through the activation of kinases. Calcium/calmodulin-dependent protein kinase II induced ovulation in the absence of gonadotropin stimulation in the perfused rabbit ovary (Kugu *et al.* 1995). Similarly, in hCG-stimulated ovaries treated with calmodulin inhibitors, a significant decrease in ovulation efficiency was observed compared with control ovaries treated only with hCG (Kugu *et al.* 1995). Thus, ovarian calmodulin probably interacts with kinases to alter the phosphorylation state of ovarian proteins, ultimately controlling ovulation in the rabbit ovary. Calmodulin could also mediate ovulation by regulating smooth muscle contraction in the follicle wall. The theca externa of quail and hamster follicles contains smooth muscle-like cells and calmodulin has been localized to chicken thecal cells, smooth muscle bundles, and fibroblasts of large follicles (Yoshimura *et al.* 1990).

Finally, calmodulin has also been implicated in the regulation of steroidogenesis (Alila *et al.* 1990, Johnson & Tilly 1990, van der Kraak 1991) and final oocyte maturation (Wasserman & Smith 1981, Bornslaeger *et al.* 1984, Sato 1990) in various vertebrates. Thus, increases in calmodulin during progesterone stimulation may be related to other ovarian processes besides ovulation.

PMAP	MAVTTTRLTWLHEKILQNHFGGKRLSLLYKGSVHGFRNGVLLDRCCNQGP TLTVIYSEDHIIGAYAEESYQEGKYASIIILFALQD TKISEWKLGLCTPETL	100
HUMAN		
PMAP	-----MGNHSSTVNVSGSCHVGTIYMRAARSFTVSEPWKRKISWGDNARDLQYVKEYKPGNKDKIKHVRILLYGPDGAGKS	74
Con	FCCDVTKYNSPTNFQIDGRNRKIVMDLKT MENLGLAQNCITISIDYEVPRCEDSLDERKIKGVIELRKSLLSALRTYEPYGSLSVQIRILLGLPTGAGKS	200
	-----Y-----K-----L-----Y-P-----RILL-GP-GAGKS	
PMAP	SFIKSVCSVIQGRVTTDAL--TSDKSYNGTYETFKIRKEGNSETFCHVFVNDVMDLKDGDICADDIKLALKGHVKEGYQKFPSSPLSPEDPGYNPRPSA	172
HUMAN	SFPNSVRSVFPQGHVTHQALVGTNTTIGSEKYRTYSIRDGKDGKYLFPILCDSLGLSEKEGGCRDDIFYILNGNIRDYQFNPMEISIKLNHHYIDPSPL	300
Con	SF--SV-SV-QG-VT--AL--T-----Y-T--IR-----G-C-DDI--L-GN----YQF-P---I-----Y---PS-	
PMAP	DDKVHVLVCVLSADSAGI-TDSVLKKMADIREAASELGIPQMAIGTHIDSACPETKKDLKNVYKSQLKQKVKDFSAAVGVFPENYIFPVKNYSDETHIND	271
HUMAN	KDRHCVAVFVDASSIQYFSSQMIVKIKRIQRELNVAGVVHALLTHVDSMDLITKGDLEIERCEPVRSKLEEVQRKLGFAISVVSNNYSSEWELDF	400
Con	-D--H---V--A-S-----K--I-----G---A--TH-DS---TRGDL-----K-----G-----I--V-NYS-E-----	
PMAP	DVNTLILSALRQMISLGDNFIEK-----	294
HUMAN	VKDVILSALRRMLWAADDPLFDLPPEQIGNLREETINCAQGGK-----	444
Con	----LILSALR-M-----D-F-E-----	

FIGURE 10. Amino acid alignment of PMAP-1 (PMAP) with human MAP-p44 (HUMAN). Deduced protein sequences for PMAP-1 and human MAP-p44 (Takahashi *et al.* 1990) were aligned using MACAW. A consensus sequence (Con) in which both sequences share identical residues is provided. Amino acids are numbered on the right and correspond to individual proteins.

Perch microtubule aggregate protein-like cDNA

Using a 450 bp upregulated cDNA obtained from DDPCR, a 1111 bp cDNA (PMAP-1; GenBank Accession No. AF085251) was obtained by library screening. PMAP-1 encodes for an 882 bp open reading frame with the presumed methionine initiation site occurring at nucleotides 19–21 and termination occurring at nucleotides 901–903 (Fig. 9). Although the sequence flanking the proposed methionine initiation site conforms well with Kozak sequences, it is possible that translation is initiated further upstream beyond the boundary of this clone since the reading frame is open upstream of the proposed ATG start site. However, it is likely that PMAP-1 is the full-length cDNA, since library screening did not yield larger cDNAs and since PMAP-1 hybridizes with a transcript on Northern blots that is nearly identical in size (see below). Assuming that the initiation site is at nucleotide 19, PMAP-1 has an 18 bp 5' untranslated region and a 211 bp 3' untranslated region.

The deduced amino acid sequence of PMAP-1 shows similarity to only two proteins in GenBank: human and chimpanzee microtubule aggregate protein, MAP-p44 (Takahashi *et al.* 1990) (Fig. 10). The presumed protein encoded by PMAP-1 is 29.6% identical and 58.5% similar to chimpanzee MAP-p44. The PMAP-1 protein does not appear to have a signal sequence, indicating that the protein is not secreted.

A single 1.1 kb transcript was detected on all Northern blots assayed. Transcript levels for PMAP significantly ($P=0.012$) increased in the presence of 17,20-P after 32 h of incubation in all fish assayed (Fig. 11A). In two fish assayed for temporal expression, PMAP levels were elevated following 32 h with 17,20-P (Fig. 11B). By 42 h, transcript levels decreased in both fish (Fig. 11B). While it appears from this figure that PMAP transcripts increase by 12 h, it should be noted that in the other fish assayed, no upregulation was observed at 12 h (data not shown). From this temporal expression pattern, it seems likely that PMAP mRNA levels increase by at least 32 h and then decline following ovulation.

High transcript levels of PMAP transcripts were detected in the gills, liver, and ovary while very low levels of transcript were detected in the trunk kidney (Fig. 11C). MAP-p44 has only been studied in the liver (Honda *et al.* 1990, Takahashi *et al.* 1990), and no localization studies have been completed to determine MAP-p44 tissue distribution in mammals.

FIGURE 11. Northern blots of PMAP transcripts in the ovary (A and B) and in other perch tissues (C). Each lane represents 0.5 µg mRNA. (A) Expression of PMAP mRNA at 32 h under 17,20-P (0.1 µg/ml, S) or ethanol vehicle (1 µl/ml, C) in the ovaries from five fish (F1–F5). The 1.1 kb transcript was upregulated by 17,20-P in all five fish assayed ($P=0.012$; pair-wise *t*-test). (B) Representative Northern blot of the temporal expression pattern of PMAP ovarian transcripts in one fish. Numbers above the lanes represent hours of incubation in Cortland medium with 17,20-P (0.1 µg/ml, 17,20P) or ethanol vehicle (1 µl/ml, Control). One other fish assayed showed a similar pattern of expression. (C) PMAP transcripts in other perch tissues. Abbreviations as in Figs 3 and 6.

Microtubule aggregate protein-p44 is expressed in human and chimpanzee hepatocytes infected with hepatitis C or D (Honda *et al.* 1990, Takahashi *et al.* 1990) and it can be induced by interferons (Kitamura *et al.* 1994). This protein associates with microtubules; however, its specific biological function remains unknown. Given that the PMAP-1 protein shares some similarity to these proteins, it may interact with microtubules in a manner similar to that described for MAP-p44 in humans and chimpanzees. However, as with MAP-p44, the ultimate function in the ovary remains unknown.

In conclusion, several progesterin-upregulated cDNAs have been isolated from the yellow perch ovary just prior to ovulation. These include perch forms of neprilysin, a lysyl oxidase type protein, calmodulin and a microtubule aggregate protein. To our knowledge, this is the first report that specifically describes progesterin-upregulated mRNAs in the vertebrate ovary at ovulation. While calmodulin has already been strongly implicated in the process of ovulation, neprilysin and microtubule aggregate protein have never been associated with ovulation or the vertebrate ovary. Finally, the characteristics of the perch lysyl oxidase expression suggest that it may be a tissue-specific or inducible form of lysyl oxidase and, therefore, different from other lysyl oxidases so far characterized.

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REFERENCES

- Ackland J, Schwartz NB, Mayo KE & Dodson RE 1992 Nonsteroidal signals originating in the gonads. *Physiological Reviews* **72** 731–787.
- Alila H, Davis J, Dowd J, Corradino R & Hansel W 1990 Differential effects of calcium on progesterone production in small and large bovine luteal cells. *Journal of Steroid Biochemistry* **36** 687–694.
- Bornslaeger E, Wilde M & Schultz R 1984 Regulation of mouse oocyte maturation: involvement of cyclic AMP phosphodiesterase and calmodulin. *Developmental Biology* **105** 488–499.
- Bradley J & Goetz FW 1994 The inhibitory effects of indomethacin, nordihydroguaiaretic acid and pyrrolidine-dithiocarbamate on ovulation and prostaglandin synthesis in yellow perch (*Perca flavescens*) follicle incubates. *Prostaglandins* **4** 11–20.
- Brannstrom M & Janson PO 1989 Progesterone is a mediator in the ovulatory process of the *in vitro*-perfused rat ovary. *Biology of Reproduction* **40** 1170–1178.
- Brannstrom M, Mayrhofer G & Robertson SA 1993 Localization of leukocyte subsets in the rat ovary during the periovulatory period. *Biology of Reproduction* **48** 277–286.
- Casey M & MacDonald C 1996 The endothelin-parathyroid hormone-related protein vasoactive peptide system in human endometrium: modulation by transforming growth factor- β . *Human Reproduction* **11** (Suppl 2) 62–82.
- Casey M, Smith J, Nagai K, Hersh L & MacDonald P 1991 Progesterone-regulated cyclic modulation of membrane metalloendopeptidase (enkephalinase) in human endometrium. *Journal of Biological Chemistry* **266** 23041–23047.
- Celio M, Pauls T & Schwaller B 1996 Introduction to EF-hand calcium-binding proteins. In *Guidebook To The Calcium-Binding Proteins*, pp 15–20. Eds M Celio, T Pauls & B Schwaller. New York: Oxford University Press.
- Chen C, Salles G, Seldin M, Kister A, Reinherz E & Shipp M 1992 Murine common acute lymphoblastic leukemia antigen (CD10 neutral endopeptidase 24-11). Molecular characterization, chromosomal localization, and modeling of the active site. *Journal of Immunology* **148** 2817–2825.
- Chien Y & Dawid I 1984 Isolation and characterization of calmodulin genes from *Xenopus laevis*. *Molecular and Cellular Biology* **4** 507–513.
- Chomczynski P 1993 A reagent for the single-step simultaneous isolation of RNA, DNA, and proteins from cell and tissue samples. *Biotechniques* **15** 532–537.
- Chomczynski P & Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Dion N, Cohen P, Crine P & Boileau G 1997 Characterization of neprilysin (EC 3.4.24.11) S2' subsite. *FEBS Letters* **411** 140–144.
- Donath J, Michna H & Nishino Y 1997 The antiovaratory effect of the antiprogesterin onapristone could be related to down-regulation of intraovarian progesterone (receptors). *Journal of Steroid Biochemistry and Molecular Biology* **62** 107–118.
- Erdos E & Skidgel R 1989 Neutral endopeptidase 24-11 (Enkephalinase) and related regulators of peptide hormones. *FASEB Journal* **3** 145–151.
- Espey LL 1980 Ovulation as an inflammatory reaction – a hypothesis. *Biology of Reproduction* **22** 73–106.
- Fischer R, Koller M, Flura M, Mathews S, Strehler-Page M, Krebs J, Penniston J, Carafoli E & Strehler E 1988 Multiple divergent mRNAs code for a single human calmodulin. *Journal of Biological Chemistry* **263** 17055–17062.
- Franklin TJ 1997 Therapeutic approaches to organ fibrosis. *International Journal of Biochemistry and Cell Biology* **29** 79–89.
- Furudate S, Masaki Y & Muto T 1994 Induction of ovulation by endothelin-3 in rats. *Journal of Endocrinology* **141** 109–112.
- Goetz F & Theofan G 1979 *In vitro* stimulation of germinal vesicle breakdown and ovulation of yellow perch (*Perca flavescens*) oocytes. Effects of 17 α -hydroxy-20 β -dihydroprogesterone and prostaglandins. *General and Comparative Endocrinology* **37** 273–285.
- Goetz F, Duman P, Berndtson A & Janowsky E 1989 The role of prostaglandins in the control of ovulation in yellow perch (*Perca flavescens*). *Fish Physiology and Biochemistry* **7** 163–168.
- Goetz FW, Berndtson AK & Ranjan M 1991 Ovulation: mediators at the ovarian level. In *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*, vol 4, pp 127–203. Eds KT Pang & MP Schreibman, New York: Academic Press, Inc.
- Green R, Lieb M, Weintraub A, Gacheru S, Rosenfield C, Shah S, Kagan H & Taubman M 1995 Identification of lysyl oxidase and other platelet-derived growth factor-inducible genes in vascular smooth muscle cells by differential screening. *Laboratory Investigation* **73** 476–482.
- Hamalainen ER, Jones TA, Sheer D, Taskinen K, Pihlajaniemi T & Kivirikko KI 1991 Molecular cloning of human lysyl oxidase and assignment of the gene to chromosome 5q23.3-31.2. *Genomics* **11** 508–516.
- Hedin L & Eriksson A 1997 Prostaglandin synthesis is suppressed by progesterone in rat preovulatory follicles *in vitro*. *Prostaglandins* **53** 91–106.
- Hellberg P, Larson L, Olofsson J, Hedin L & Brännström M 1991 Stimulatory effects of bradykinin on the ovulatory process in the *in vitro*-perfused rat ovary. *Biology of Reproduction* **44** 269–274.

- Himeno N 1986 Effect of prostaglandins on collagen synthesis in rabbit ovarian follicles during the ovulatory process. *Folia Endocrinology* **62** 1181–1193.
- Holmes PV, Sogn J, Schillinger E & Janson P 1985 Effects of high and low preovulatory concentrations of progesterone on ovulation from the isolated perfused rabbit ovary. *Journal of Reproduction and Fertility* **75** 393–399.
- Honda Y, Kondo J, Maeda T, Yoshiyama Y, Yamada E, Shimizu Y, Shikata T & Ono Y 1990 Isolation and purification of a non-A, non-B hepatitis-associated microtubular aggregate protein. *Journal of General Virology* **71** 1999–2004.
- Hooper N 1994 Families of zinc metalloproteases. *FEBS Letters* **354** 1–6.
- Hsu T & Goetz FW 1991 Synergistic induction of ovulation and prostaglandin synthesis in goldfish (*Carassius auratus*) follicles by sodium orthovanadate and hydrogen peroxide. *Biology of Reproduction* **45** 912–917.
- Johnson A & Tilly J 1990 Evidence that arachidonic acid influences hen granulosa cell steroidogenesis and plasminogen activator activity by a protein kinase C-independent mechanism. *Biology of Reproduction* **43** 922–928.
- Kenyon K, Modi WS, Contente S & Friedman RM 1993 A novel human cDNA with a predicted protein similar to lysyl oxidase maps to chromosome 15q24–q25. *Journal of Biological Chemistry* **268** 18435–18437.
- Kim Y, Boyd C & Csiszar K 1995 A new gene with sequence and structural similarity to the gene encoding human lysyl oxidase. *Journal of Biological Chemistry* **270** 7176–7182.
- Kitamura A, Takahashi K, Okajima A & Kitamura N 1994 Induction of the human gene for p44, a hepatitis-C-associated microtubular aggregate protein, by interferon- α/β . *European Journal of Biochemistry* **224** 877–883.
- Kozak M 1986 Point mutations define a sequence flanking the aug initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44** 283–292.
- van der Kraak G 1991 Role of calcium in the control of steroidogenesis in preovulatory ovarian follicles of the goldfish. *General and Comparative Endocrinology* **81** 268–275.
- Kugu K, Dharmarajan A, Preutthipan S & Wallach E 1995 Role of calcium/calmodulin-dependent protein kinase II in gonadotropin-induced ovulation in *in vitro* perfused rabbit ovaries. *Journal of Reproduction and Fertility* **103** 273–278.
- Lazarus H, Cruikshank W, Narasimhan N, Kagan H & Center D 1994 Induction of human monocyte mobility by lysyl oxidase. *Matrix Biology* **14** 727–773.
- Lipner H & Greep R 1971 Inhibition of steroidogenesis at various sites in the biosynthetic pathway in relation to induced ovulation. *Endocrinology* **88** 602–607.
- Lipner H & Wendelken L 1971 Inhibition of ovulation by inhibition of steroidogenesis in immature rats. *Proceedings of the Society of Experimental Biology and Medicine* **136** 1141–1145.
- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM & O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes and Development* **9** 2266–2278.
- Matso K, Sato K, Ikeshima H, Shimoda K & Takano T 1992 Four synonymous genes encode calmodulin in the teleost fish, medaka (*Oryzias latipes*): conservation of the multigene one-protein principle. *Gene* **119** 279–281.
- Morgan A, Keeble SC, London SN, Muse KN & Curry TE 1994 Antiprogesterone (RU486) effects on metalloproteinase inhibitor activity in human and rat granulosa cells. *Fertility and Sterility* **61** 949–955.
- Mori T, Suzuki A, Nishimura T & Kambe-gawa K 1977 Inhibition of ovulation in immature rats by anti-progesterone antiserum. *Endocrinology* **73** 185–186.
- Murdoch WJ 1998 Perturbation of sheep ovarian surface epithelial cells by ovulation: evidence for roles of progesterone and poly (ADP-ribose) polymerase in the restoration of DNA integrity. *Journal of Endocrinology* **156** 503–508.
- Murdoch WJ & Farris ML 1988 Prostaglandin E₂-9-ketoreductase activity of preovulatory ovine follicles. *Journal of Animal Sciences* **66** 2924–2929.
- Murdoch WJ & McCormick RJ 1989 Production of low molecular weight chemoattractants for leukocytes by periovulatory ovine follicles. *Biology of Reproduction* **40** 86–90.
- Murdoch WJ, Petterson TA, Van Kirk EA, Vincent DL & Inskeep EK 1986 Interactive roles of progesterone, prostaglandins, and collagenase in the ovulatory mechanism of the ewe. *Biology of Reproduction* **35** 1187–1194.
- Painter R, Dukes R, Sullivan J, Carter R, Erdos E & Johnson 1988 A function of neutral endopeptidase on the cell membrane of human neutrophils. *Journal of Biological Chemistry* **263** 9456–9461.
- Putkey J, Ts'ui K, Tanaka T, Lagace L, Stein J, Lai E & Means A 1983 Chicken calmodulin genes. A species comparison of cDNA sequences and isolation of a genomic clone. *Journal of Biological Chemistry* **258** 11864–11870.
- Saito H, Papaconstantinou J, Sato H & Goldstein S 1997 Regulation of a novel gene encoding a lysyl oxidase-related protein in cellular adhesion and senescence. *Journal of Biological Chemistry* **272** 8157–8160.
- Sato E 1990 Suppression of spontaneous maturation of isolated cumulus free mouse oocytes by a calmodulin antagonist. *Endocrinologia Japonica* **37** 19–26.
- Shipp MA, Richardson NE, Sayre PH, Brown NR, Masteller EL, Clayton LK, Ritz J & Reinherz EL 1988 Molecular cloning of the common acute lymphoblastic leukemia antigen (CALLA) identifies a type II integral membrane protein. *Proceedings of the National Academy of Sciences of the USA* **85** 4819–4823.
- Smith-Mungo L & Kagan H 1998 Lysyl oxidase: properties, regulation and multiple functions in biology. *Matrix Biology* **16** 387–398.
- Takahashi M, Ford JJ, Yoshinaga K & Greep RO 1974 Induction of ovulation in hypophysectomized rats by progesterone. *Endocrinology* **95** 1322–1326.
- Takahashi K, Kitamura N, Shibui T, Kamizono M, Matsui R, Yoshiyama Y, Maeda T, Kondo J, Honda Y, Yamada E, Shimiau Y, Teranishi Y & Nakanishi S 1990 Cloning, sequencing and expression in *Escherichia coli* of cDNA for a non-A, non-B hepatitis-associated microtubular aggregates protein. *Journal of General Virology* **71** 2005–2011.
- Tanaka K, Li ZD & Ataka Y 1987 Studies of ovulation in the perfused ovary of the fowl (*Gallus domesticus*). *Journal of Reproduction and Fertility* **80** 411–416.
- Tanaka N, Espey LL, Stacy S & Okamura H 1992 Epostane and indomethacin actions on ovarian kallikrein and plasminogen activator activities during ovulation in the gonadotropin-primed immature rat. *Biology of Reproduction* **46** 665–670.
- Tedeschi C, Hazum E, Kokia E, Ricciarelli E, Adashi E & Payne D 1992 Endothelin-1 as a luteinization inhibitor: inhibition of rat granulosa cell progesterone accumulation via selective modulation of key steroidogenic steps affecting both progesterone formation and degradation. *Endocrinology* **131** 2476–2478.
- Theofan G & Goetz FW 1981 The *in vitro* effects of actinomycin D and cycloheximide on germinal vesicle

- breakdown and ovulation of yellow perch (*Perca flavescens*) oocytes. *Comparative Biochemistry and Physiology* **69A** 557–561.
- Tsafrii A, Abisogun AO & Reich R 1987 Steroids and follicular rupture at ovulation. *Journal of Steroid Biochemistry and Molecular Biology* **27** 359–363.
- Wasserman W & Smith D 1981 Calmodulin triggers the resumption of meiosis in amphibian oocytes. *Journal of Cell Biology* **89** 389–394.
- Wolf K & Quimby M 1969 Fish cell and tissue culture. In *Fish Physiology*, vol 3, pp 253–305. Eds W Hoar & D Randall. New York: Academic Press, Inc.
- Yoshimura Y, Hosoi Y, Atlas SJ, Bongiovanni AM, Santulli R & Wallach EE 1986 The effects of ovarian steroidogenesis on ovulation and fertilizability in the *in vitro* perfused rabbit ovary. *Biology of Reproduction* **35** 943–948.
- Yoshimura Y, Hosoi Y, Bongiovanni AM, Santulli R, Atlas SJ & Wallach EE 1987 Are ovarian steroids required for ovum maturation and fertilization? Effects of cyanoketone on the *in vitro* perfused rabbit ovary. *Endocrinology* **120** 2555–2561.
- Yoshimura Y, Okamoto T & Tamura T 1990 Immunohistochemical observations on the localization of calmodulin in the chicken ovary. *Japanese Poultry Science* **27** 356–362.
- Zar J 1996 *Biostatistical Analysis*. New Jersey: Prentice Hall.

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