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

D M Langenau, F W Goetz and S B Roberts
University of Notre Dame, Department of Biological Sciences, Notre Dame, Indiana 46556, USA
(Requests for offprints should be addressed to F W Goetz)

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, 17α,20β-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 µg/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.

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 aminogluthethimide or cyano-ketone (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 PGE2-9-ketoreductase, thereby increasing the levels of PGF at ovulation. In support of this, isoxazol decreases the activity of PGE2-9-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, 17α,20β-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 µl 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 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 (RNAmap; 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 (API through API5; GenHunter Corp.) in the presence of [α-33P]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 glycerogen 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 (Qiagen II; Qiagen, Chatsworth, CA, USA). Gel purified DNA was ligated into pCRII (In Vitrogen, San Diego, 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.

Nyロン 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 Xhol/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
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 (opisthnockphos), 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 progestin-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.
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 conserved domain structures (cytoplasmic domain (<<<CYTOPLASMIC DOMAIN<<<L), membrane spanning region (T>MEMBRANE SP DOM<A), and extracellular domain (T>>>EXTRACELLULAR DOMAIN>>>) 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.

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.
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 progestin 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\(\beta\) (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 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
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 ovolatory 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,
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, 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).
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

![Northern blots of PLO transcripts in the ovary (A and B) and in other perch tissues (C). Each lane represents 0·5 µg mRNA. (A) Expression of PLO 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 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 µg/ml, 17,20P) or ethanol vehicle (1 µl/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.](image)
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.

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

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.

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.
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.

**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 (***)

**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 progestin-upregulated cDNAs have been isolated from the yellow perch ovary just prior to ovulation. These include perchn forms of neprilsin, a lysyl oxidase type protein, calmodulin and a microtubule aggregate protein. To our knowledge, this is the first report that specifically describes progestin-upregulated mRNAs in the vertebrate ovary at ovulation. While calmodulin has already been strongly implicated in the process of ovulation, neprilsin 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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