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Expression of Avian Urokinase and Tissue-Type Plasminogen Activator Messenger Ribonucleic Acid during Follicle Development and Atresia[1]

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ABSTRACT

While several studies have documented the presence of plasminogen activator (PA) activity in hen ovarian follicle granulosa and theca tissues, to date it has not been possible to conclusively distinguish between the urokinase (u) and the tissue-type (t) form of the enzyme; this inability is due, in part, to lack of the cloned or characterized chicken tPA gene or gene product. Thus, the present studies were conducted to identify a partial cDNA for chicken tPA and subsequently to evaluate expression of uPA and tPA mRNA in granulosa and theca tissues in vivo and in vitro. Urokinase PA mRNA levels were highest in prehierarchical-follicle granulosa (3- to 5- and 6- to 8-mm follicles) and theca (6- to 8-mm follicles) tissue compared to hierarchical (9–12 mm through largest preovulatory) follicles. In vitro treatment with a phorbol ester (phorbol 12-myristate, 13-acetate), but not a cAMP analogue (8-bromo-cAMP), significantly increased uPA mRNA levels in both granulosa and theca tissue from the largest and second-largest preovulatory follicles. Of special interest was the finding that levels of uPA mRNA were 10.9-fold higher in atretic compared to morphologically normal 3- to 5-mm follicles. Moreover, 4- to 8-mm-follicle granulosa cells, which spontaneously undergo apoptosis in vitro, demonstrated a rapid increase in uPA mRNA levels after 1 h of incubation (prior to the detection of oligonucleosome formation) while levels in preovulatory-follicle granulosa cells, which do not undergo spontaneous apoptosis, were not altered after 18 h of incubation. By contrast, while tPA mRNA can be identified in granulosa and theca tissues from prehierarchical and preovulatory follicles following polymerase chain reaction amplification, constitutively expressed levels of the transcript were too low to reliably measure by Northern blot analysis. These data indicate that while the chicken expresses a tPA gene that is homologous to the mammalian tPA, uPA is the predominant PA expressed in the hen ovary. In addition, the higher levels of uPA mRNA found in granulosa cells actively undergoing apoptosis and in follicles most susceptible to atresia (4–8 mm) suggest a role for this protease in mediating processes both during the early stages of programmed cell death and in the later stages of follicle atresia and resorption.

INTRODUCTION

Plasminogen activators are serine proteases that convert the inactive zymogen, plasminogen, to the endopeptidase, plasmin. It is proposed that the mammalian urokinase (u) plasminogen activator (PA) system plays a role in angiogenesis, mammary gland involution, and embryo implantation while tissue-type (t) PA is critical to the process of fibrinolysis and is associated with a number of ovarian processes requiring extracellular proteolysis, such as cumulus cell expansion, germinal vesicle breakdown, meiotic maturation, and ovulation [1–8]. Moreover, there are considerable data to indicate that PAs play an important role in tumor cell invasion and metastasis [9, 10].

Mammalian uPA and tPA enzymes are the products of separate genes and represent immunologically distinct proteins (e.g., tPA, [11, 12]; uPA, [13, 14]). The chicken uPA gene has previously been cloned [15], and activity of this enzyme has been implicated in processes such as cell migration and tissue remodeling during embryonic development and in the bursa of fabricius [16, 17]. PA activity in the hen ovary has previously been studied by several groups of investigators (e.g., [18–22]). In general, granulosa cell PA activity is increased by pharmacologic activators of protein kinase C (e.g., phorbol esters), growth factors (e.g., epidermal growth factor, transforming growth factors α and β), or prostaglandin (PGE_2), and it is suppressed by activators of protein kinase A or calcium mobilization (e.g., LH, vasoactive intestinal peptide, forskolin, cAMP analogues, calcium ionophore) [18, 20, 21, 23, 24]. However, while uPA immunoreactive protein has been identified within the granulosa cell layer [22], direct evidence for a tPA-like protease in any chicken tissue has been absent, due, in large part, to the lack of the cloned chicken tPA gene and/or characterized protein. Only on the basis of indirect enzymatic assays has it has been concluded that tPA activity is expressed within hen ovarian tissues [25].

Moreover, in light of results from nonovarian tissues indicating a relationship between programmed cell death and activation of proteolytic enzymes [26, 27], it was of significance to establish the relationship between the expression of PA mRNA and follicle atresia, which is mediated via the process of apoptosis [28]. With regard to the latter, we have recently characterized a population of apoptosis-sensitive granulosa cells that are derived from prehierarchical follicles ($\leq$ 8 mm in diameter) as well as apoptosis-resistant granulosa derived from preovulatory follicles [29]. Therefore, the objectives of the present studies were to determine whether there exists a tPA-like gene expressed within the hen ovary and to evaluate the relative expression of uPA and tPA during follicle development and atresia as well as with the progression of programmed cell death in granulosa cells.

MATERIALS AND METHODS

Animals and Reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–40 wk of age and laying regular sequences of at least 5–6 eggs, were used in all studies described. Birds were housed individually in laying batteries, provided free access to feed (Purina Layena Mash; Purina Mills, St. Louis, MO) and water, and exposed to a photoperiod of

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15L:9D with lights-on at 0000 h. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed by cervical dislocation approximately 16–18 h prior to a midsequence ovulation. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Oligonucleotide primers (for polymerase chain reaction [PCR] amplification and sequencing) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) or the University of Notre Dame Biotechnology Core Facility. Additional reagents were acquired from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from granulosa and theca tissue from the largest (F1), second-largest (F2), and third-largest (F3) preovulatory follicles, from follicles recently selected into the follicle hierarchy (9- to 12-mm follicles), from prehierarchical (6–8 mm and 3–5 mm) follicles, and from stromal tissue as previously described [30, 31]. In addition, morphologically normal and atretic follicles (3- to 5-mm diameter) were collected and processed without separation of granulosa and theca layers. Atretic follicles were identified on the basis of the presence of follicle haemorrhagia, collapsed morphology, and opaque appearance [29]. Total cellular RNA from tissue at each stage of development was isolated using Trizol Reagent (Gibco-BRL, Gaithersburg, MD), and purity and quantity were assessed by measuring the optical density of each sample at 260 nm vs. 280 nm. Total cellular RNA (15 μg) was electrophoresed on 1% agarose gels in the presence of formaldehyde [32] and then transferred to nitrocellulose Nitro ME membranes (MSI, Westboro, MA) by capillary action overnight. Poly(A)$^+$-enriched RNA was obtained using the Micro-FastTrack mRNA isolation system (Invitrogen, San Diego, CA) according to manufacturer recommendations.

Northern blot analysis using riboprobes was conducted as previously described [32]. Antisense uPA RNA probe was synthesized by in vitro transcription from a linearized (*Xho* I digested) 1.3-kilobase (kb) uPA plasmid template using T3 RNA polymerase (Promega, Madison, WI) also T7 RNA below), [α^{32}P]CTP (3000 Ci/mmol; Amersham, Arlington Heights, IL), and the Gemini II Riboprobe Core System (Promega). The tPA cRNA probe was synthesized from a *Xho* I-linearized template (prepared in pBluescript vector as described below) using T7 RNA polymerase (Promega). All blots were prehybridized for 4 h; hybridizations were conducted overnight with the cRNA probes (specific activity approximately 10^9 cpm/μg; 2×10^6 cpm/ml hybridization buffer) and subsequently washed under highly stringent conditions at 65°C. Membranes were exposed to autoradiographic film at −70°C for 2–5 days or to phosphorimaging plates for analysis by a PhosphorImager 445 SI system (Molecular Dynamics, Sunnyvale, CA) for 1–7 days.

Granulosa Cell and Theca Tissue Incubations

Granulosa cells and theca tissue were collected from preovulatory (F1 and F2) follicles and dispersed in 0.3% collagenase (type 2; Worthington, Freehold, NJ) as previously described [30]. It should be noted that while the granulosa cell preparation is a pure population of cells, the theca layer consists of a variety of cell types, including fibroblasts and some red and white blood cells (but not granulosa cells). Cells were incubated as suspension cultures in the absence or presence of treatments in 1 ml (2.5×10^6 cells) medium 199 supplemented with Hanks' salts (Gibco-BRL) without serum (M199/Hepes) for 18 h at 37°C in a shaking water bath. Treatments for both tissue types included medium alone (incubated control), 1 mM 8-bromo-cAMP (8-br-cAMP), or 167 nM phorbol 12-myristate, 13-acetate (PMA). These treatments and doses were selected for use on the basis of their ability to induce (PMA) or inhibit (8-br-cAMP) hen granulosa cell PA activity [18]. After incubation, cells were gently pelleted by centrifugation (200 × *g*; 20°C; 5 min), media were discarded, and cells were frozen (−70°C) until RNA was collected.

An additional set of studies was conducted to establish the temporal relationship between the onset of apoptotic cell death in granulosa cells and expression of uPA mRNA. Granulosa cells from a pool of 20–24 follicles, 4 to 8 mm, were collected [30], dispersed, and frozen immediately (T0 control) or were incubated as suspension cultures in M199/Hepes as described above for 0.5, 1, 2, 3, or 6 h, or for 2 or 3 h in M199/Hepes containing 1 mM 8-br-cAMP. In a parallel study, dispersed granulosa cells from F1 and F2 follicles were incubated in M199/Hepes alone for 1, 3, 6, or 18 h or treated with the ceramide analogue, *N*-octanoyl-sphingosine (C8-ceramide; Biomol, Plymouth Meeting, PA), for 3 or 6 h. C8-ceramide has recently been shown to induce apoptosis in hierarchical-follicle granulosa cells within a 6-h treatment interval [33]. After incubation, cells were pelleted and frozen at −70°C until processed for the collection of DNA or RNA.

DNA Analysis for Oligonucleosomes

Genomic DNA was extracted and analyzed for internucleosomal cleavage, indicative of apoptosis, as previously described [29]. Briefly, DNA samples (1 μg/sample) were 3′ end-labeled with [α^{32}P]dideoxy-ATP (3000 Ci/mmol; New England Nuclear; NEN, Boston, MA) using terminal transferase (Boehringer Mannheim, Indianapolis, IN). Labeled DNA was resolved by electrophoresis through 2% agarose gels; then gels were dried and exposed to x-ray film overnight. Quantification of oligonucleosomes was performed by excision and β-scintillation counting of low molecular weight (< 20 kb) DNA.

Isolation and Characterization of a Partial Chicken tPA cDNA

Total cellular RNA was isolated from F1 theca tissue and reverse transcribed into first-strand cDNA using oligo(deoxythymidine) primer and recombinant M-MLV (Moloney murine leukemia virus) reverse transcriptase in the presence of deoxynucleotides (Perkin-Elmer, Norwalk, CT). Sequences for oligonucleotide primer pairs were based on the published sequence for the human tPA cDNA [11]: forward primer, TACTGCAGAAACCCAGA (base pairs [bp] 615–631); reverse primer, GTATGTTCTGCCCAAGA (bp 1212–1196).

First-strand cDNA was subjected to 35 cycles of PCR amplification using GeneAmp core reagents (Perkin-Elmer; 2-min denaturation at 94°C on the first cycle and 1 min per cycle thereafter; 1 min annealing at 55°C; and 1 min extension at 72°C for the first 34 cycles, 7 min extension on the final cycle). Amplified 607-bp PCR products (572 bp chicken tPA plus primer sequence) were resolved through a 1.2% agarose gel, isolated, purified (GeneClean; Bio 101,

La Jolla, CA), and subcloned into the pBluescript vector (Stratagene, La Jolla, CA) for large-scale plasmid preparation. Nucleic acid sequence analysis was conducted in both the forward and reverse directions from two different clones by the dideoxychain termination method using [^{35}S]dATP (Amersham) and the Sequenase version 2.0 sequencing kit (U.S. Biochemicals, Cleveland, OH). Sequence data were assembled and analyzed using the MacVector and AssemblyLIGN programs (IBI, New Haven, CT).

Analysis of tPA Expression by Southern Blot

Southern blot analysis was conducted to evaluate expression of tPA following reverse transcription (RT) and PCR of total cellular RNA collected from F1 and 6- to 8-mm-follicle granulosa and theca tissue as well as stromal tissue. Primers and conditions for RT-PCR were as described above; amplified samples included the tPA forward and reverse primers plus polymerase without cDNA, and cDNA plus polymerase without primers as negative controls. One half of the resulting PCR reaction was subjected to a restriction digest with *Bam*HI (20 U; Promega). On the basis of the newly established chicken tPA cDNA sequence, it was determined that such a digest of the amplified 607-bp product would produce a 510-bp fragment that would aid in the subsequent verification of amplified tPA.

Both undigested and digested DNA were run on a 1.2% agarose gel and then transferred to a Zetabind membrane (CUNO, Inc., Meriden, CT) by capillary action overnight. Southern blots were probed using the verified chicken tPA cDNA labeled with [^{32}P]dCTP (Amersham) by means of the Megaprime DNA labeling system (Amersham). Briefly, blots were denatured (0.5 N NaOH, 1 M NaCl) and neutralized (0.5 M Tris, 1.5 M NaCl, pH 7.5) for 30 min each; they were then prehybridized in 5-strength sodium chloride-sodium citrate solution (SSC; single-strength SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0), 10-strength Denhardt's solution, 50 mM $NaPO_4$ (pH 6.7), 5% dextran sulfate, and 50% deionized formamide containing 0.5 mg/ml denatured herring sperm (Promega) in a Hotshaker (Bellco, Inc., Vineland, NJ) at 42°C for 18 h. Hybridization was accomplished at 42°C for 20 h in 5-strength SSC, single-strength Denhardt's, 20 mM $NaPO_4$ (pH 6.7), 10% dextran sulfate, and 50% formamide containing 0.1 mg/ml denatured herring sperm. Blots were stringently washed with double-strength SSC containing 0.1% SDS for 15 min at 20°C and with 0.1-strength SSC plus 0.1% SDS for 15 min at 20°C; they were then washed twice for 30 min each time with 0.1-strength SSC plus 0.1% SDS at 60°C before exposure to x-ray film.

Data Analysis

Northern blot analyses were repeated a minimum of three times. The relative extent of hybridization was evaluated either by densitometry (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ) or by the ImageQuant data reduction system (Molecular Dynamics). Equal loading of RNA onto gels was verified after the evaluation of ethidium bromide-stained 28S and 18S bands of ribosomal RNA. The rationale for this analysis is that β-actin mRNA, a common housekeeping gene, is not expressed at consistent levels during hen follicle development (unpublished results). Moreover, it is reasoned that potential bias due to uneven loading, incomplete transfer, and/or unequal hybridization efficiency within a blot is virtually eliminated by combining data from several independent, replicate analyses. Messenger RNA levels (fold-difference in relation to the appropriate control) are presented as the mean ± SEM of scanning data from replicate experiments. Data were analyzed by a one-way analysis of variance, and significant interactions were determined using Student's *t*-test or the Fisher least-significant difference multiple range test.

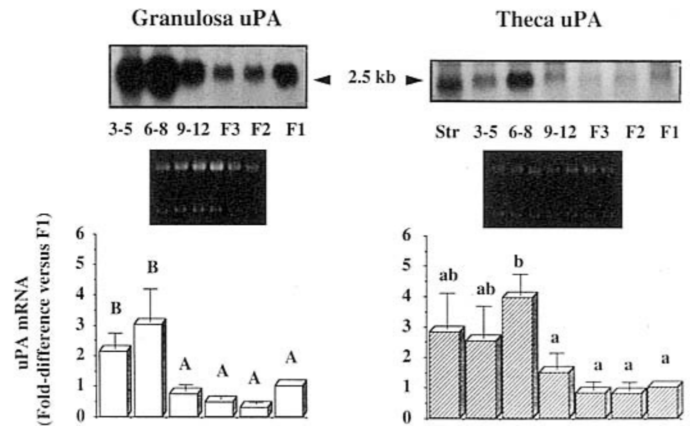

FIG. 1. Representative Northern blots of uPA mRNA from granulosa and theca tissue during follicle development (upper panels), ethidium bromide-stained gels (center panels), and summary of densitometric scanning data (lower panels; mean densitometric units expressed as fold-difference compared to F1 [n = 4 replicate experiments]). Autoradiographs of granulosa and theca tissue were exposed for equal lengths of time. Str, stromal tissue. A,B; a,b: mean ± SEM, $p < 0.05$.

RESULTS

Urokinase PA mRNA Levels during Follicle Development and after Treatments In Vitro

Highest levels of uPA mRNA in granulosa cells were found in 3- to 5-mm and 6- to 8-mm prehierarchical follicles, while levels were significantly lower in granulosa from 9- to 12-mm through F1 follicles ($p < 0.05$; Fig. 1, left panels). A similar trend for a decrease in uPA mRNA was observed in theca tissue during development, except that levels of uPA mRNA in only the 6- to 8-mm follicles were significantly higher than those from 9- to 12-mm through F1 follicles ($p < 0.05$; Fig. 1, right panels). A comparison of constitutive uPA mRNA expression in the two follicle tissue types indicates approximately 5-fold (in preovulatory follicles) to 10-fold (prehierarchical follicles) higher levels in the granulosa vs. the theca layer. Urokinase PA mRNA levels were increased in both preovulatory (F1 and F2)-follicle granulosa cells and theca tissue after an 18-h incubation with PMA ($p < 0.05$), but not cAMP, as compared to levels in incubated controls (Fig. 2).

Urokinase PA mRNA with Relation to Follicle Atresia and Granulosa Cell Apoptosis

Urokinase PA mRNA levels were significantly higher in atretic compared to morphologically normal 3- to 5-mm follicles (mean increase, 10.9 ± 3.4-fold, $p < 0.05$; Fig. 3). In view of these markedly higher levels detected during follicle atresia, short-term incubations of granulosa cells were conducted to test the hypothesis that elevated expression of uPA mRNA is related to the onset of apoptotic cell death in granulosa cells. Granulosa cells from 4- to 8-mm follicles incubated in M199/Hepes were found to express elevated levels of uPA mRNA associated with increasing

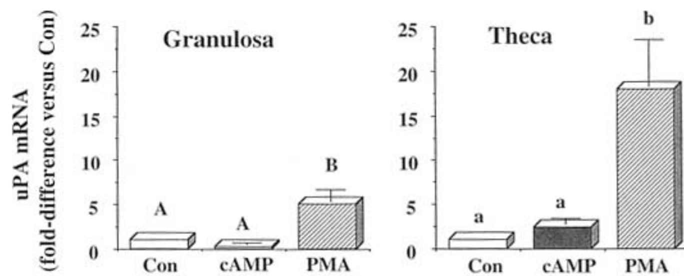

FIG. 2. Summary of densitometric scanning data from 18-h incubations of preovulatory-follicle granulosa cells and theca tissue in the absence and presence of 8-br-cAMP (cAMP; 1 mM) or PMA (167 nM). Data represent the fold-change in uPA mRNA levels vs. level in the appropriate 18-h control (Con) from three replicate experiments. A,B; a,b: mean ± SEM, $p < 0.05$.

length of incubation ($p < 0.05$ at T1 vs. T0; Fig. 4A); this increase was not inhibited by incubation of cells in the presence of 1 mM 8-br-cAMP ($p > 0.05$ at T2 and T3). The spontaneous increase in uPA mRNA levels in 4- to 8-mm-follicle granulosa cells occurred before detection of significant oligonucleosome formation was possible (Fig. 5). By comparison, no time-dependent increase in uPA mRNA was detected in preovulatory-follicle granulosa cells incubated in M199/Hepes after 18 h of incubation, and treatment with C8-ceramide failed to increase uPA mRNA levels ($p > 0.05$ at T3 and T6; Fig. 4B). Additional aliquots of cells concomitantly treated with C8-ceramide and processed for DNA verified the presence of oligonucleosome formation by 6 h of incubation (data not shown), as previously determined [33].

Identification and Characterization of a Chicken tPA cDNA

The nucleic acid and deduced amino acid sequences for the chicken tPA partial cDNA are presented in Figure 6A. The amplified product corresponds to part of the first kringle region, the entire second kringle region, and a portion of the catalytic domain as compared to mammalian tPA (Fig. 6B). A comparison of sequences determined 76.9% and 80.2% nucleic acid homology and 75.2% and 76.7% amino acid identity vs. the comparable region of the human [11] and rat [12] tPA sequences, respectively (Table 1, Fig. 7). By contrast, the chicken tPA nucleic acid and amino acid identity to chicken uPA [15] is calculated to be 54.9% and 35.1%, respectively.

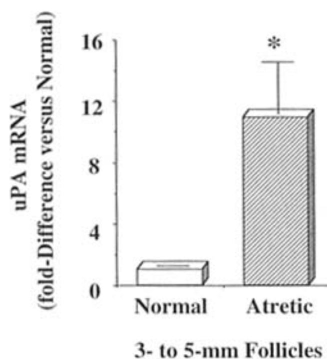

FIG. 3. Levels of uPA mRNA from atretic follicles expressed as a fold-increase compared to morphologically normal 3- to 5-mm follicles (n = 4 replicate blots). *: Mean ± SEM, $p < 0.05$.

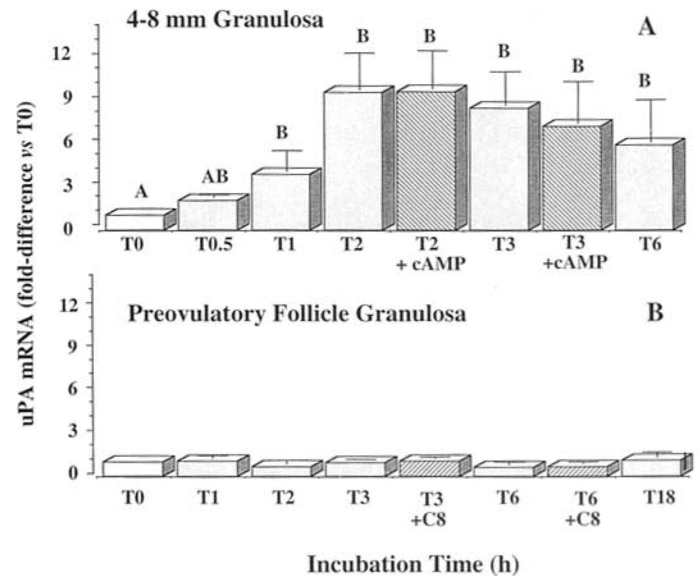

FIG. 4. **A**) Levels of uPA mRNA levels from prehierarchical (4–8 mm)-follicle granulosa cells incubated for various lengths of time in the absence or presence of 8-bromo-cAMP (cAMP), expressed as a fold-increase compared to levels in unincubated (T0) cells (n = 3–4 replicate experiments). **B**) uPA mRNA from preovulatory-follicle granulosa cells incubated for up to 18 h in the absence or presence of the ceramide analogue, C8-ceramide (C8), expressed as a fold-difference compared to value at T0 (n = 4–6 replicate experiments). A,B: mean ± SEM, $p < 0.05$.

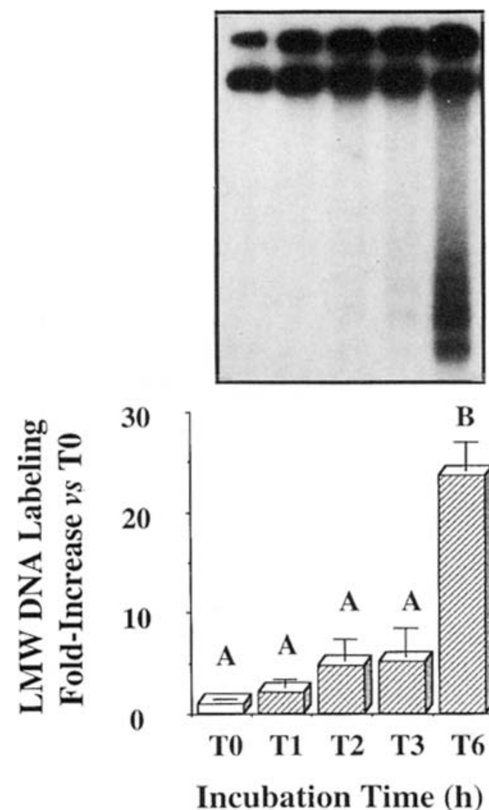

FIG. 5. Oligonucleosome formation during the early stages of spontaneous apoptotic cell death in 4- to 8-mm-follicle granulosa cells incubated in serum-free M199/Hepes. Top panel, representative autoradiograph; bottom panel, fold-increase in [^{32}P]dideoxy-ATP-labeled low molecular weight (LMW) DNA compared to value in T0 control cells (n = 4 replicate experiments). A,B: mean ± SEM, $p < 0.05$.

A

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1     <C CAA GAC TCA AAG CCC TGG TGC TAC GTC TTC AAG GCA GGG AAA TAC    46
1        Gln Asp Ser Lys Pro Trp Cys Tyr Val Phe Lys Ala Gly Lys Tyr    15

                                           <- K1  K2 ->
47    ATC TCT GAG TTC TGC AGC ACT CCA GCC TGC ACT AAG GTT GCA GAA GAA   94
16    Ile Ser Glu Phe Cys Ser Thr Pro Ala Cys Thr Lys Val Ala Glu Glu   31

95    GAT GGG GAC TGC TAC ACT GGA AAC GGG CTG GCA TAC CGC GGC ACC CGC  142
32    Asp Gly Asp Cys Tyr Thr Gly Asn Gly Leu Ala Tyr Arg Gly Thr Arg   47

143   AGC CGC ACC AAG TCT GGG TTC TCC TGC CTC CCG TGG AAT CCC GTG TTC  190
48    Ser Arg Thr Lys Ser Gly Phe Ser Cys Leu Pro Trp Asn Pro Val Phe   63

191   CTA ACG AGC AAG ATT TAC ACC GCC CTG GAA GAG CAA CGA CGG GCC CTG  238
64    Leu Thr Ser Lys Ile Tyr Thr Ala Leu Glu Glu Gln Arg Arg Ala Leu   79

239   GGC CTC GGG AAG CAC AAT CAC TGC AGG AAT CCC GAT GGG GAT GCC CAG  286
80    Gly Leu Gly Lys His Asn His Cys Arg Asn Pro Asp Gly Asp Ala Gln   95

287   CCT TGG TGC CAC GTG TGG AAG GAC CGT CAG CTT ACC TGG GAG TAC TGT  334
96    Pro Trp Cys His Val Trp Lys Asp Arg Gln Leu Thr Trp Glu Tyr Cys  111

335   GAT GTG CCC CAG TGC GTC ACC TGT GGC CTG AGA CAG TAC AAG CGG CCC  382
112   Asp Val Pro Gln Cys Val Thr Cys Gly Leu Arg Gln Tyr Lys Arg Pro  127

                  Catalytic  Domain  ->
383   CAG TTC CGC ATC AAA GGA GGC CTC TTC GCC GAC ATC ACC TCC CAC CCC  430
128   Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Thr Ser His Pro  143
                 ^
431   TGG CAG GCC GCC ATC TTC GTC AAG AAC AGG CGT GCC CCG GGA CAG CGG  478
144   Trp Gln Ala Ala Ile Phe Val Lys Asn Arg Arg Ala Pro Gly Gln Arg  159

479   TTT CTG TGT GGG GGG ATC CTG ATC AGC TCC TGC TGG GTG CTG TCA GCC  526
160   Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Val Leu Ser Ala  175

527   GCC CAC TGC TTC CAG GAG AGG TAC CCG CCT CAC CAT CTC ACG GTG A>   572
176   Ala His Cys Phe Gln Glu Arg Tyr Pro Pro His His Leu Thr Val      190
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B

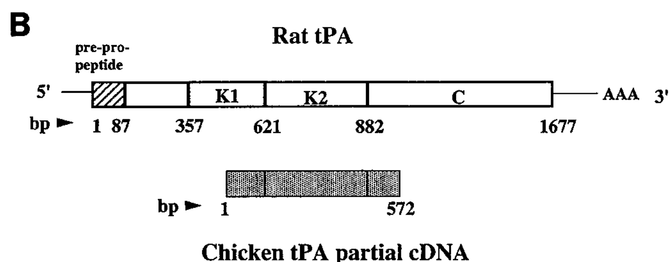

FIG. 6. **A**) Nucleic acid and deduced amino acid sequences for the partial chicken tPA cDNA. The first amino acid listed corresponds to residue 152 from the published rat tPA sequence (12). Single underlined region represents a portion of kringle domain 1 (K1), while double-underlined region corresponds to kringle domain 2 (K2). ∧ indicates the peptide bond cleaved to form two-chained tPA. **B**) Schematic diagram of the chicken tPA PCR products aligned to the rat tPA cDNA.

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c tPA   QDSKPWCYVF KAGKYISEFC STPACTKVAE EDGDCYTGNG LAYRGTRSRT KSGFSCLPWN   60
h tPA   R                S           ---S  GNS   F    S     H L  E  A      I
r tPA   R V              TT          P GPT  --   V K  VT    H F  T KA
                           C   E                                      D
c uPA   CLNGGTCITY RFFSQ KRL  PGYG -EI-D TNSI  S    ED   -MA-E DP --   Y H

c tPA   PVFLTSKIYT ALEEQRRALG LGKHNHCRNP DGDAQPWCHV WKDRQLTWEY CDVPQCVTCG  120
h tPA   SMI  IG V    QTPSAQ          Y         K      L N R           S S
r tPA   SMI  IG T    WRANSQ       R  Y         K      M   K         MSP S
                                                                   S    E
c uPA    SVIRWGD H   DLKNALQ         Y      N RSR   YT KRRYSIQETP  TIEK R

c tPA   LRQYKRPQFR IKGGLFADIT SHPWQAAIFV KNRRAPGQRF LCGGILISSC WVLSAAHCFQ  180
h tPA      SQ                A         A  H  S  E              I
r tPA       Q            T               K S  E      V                V
c uPA   Q SFSK-Y K  V  SQ EVE TQ  I G  - Q IMGTD -    S  DP      T     Y

c tPA   ERYPPHHLTV  190
h tPA     F
r tPA     F    K
c uPA   NPTKKQPNKS
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FIG. 7. Comparison of the deduced chicken tPA amino acid sequence with the human and rat tPA and chicken uPA sequences. Blank spaces within aligned sequences represent residues similar to those in the chicken tPA sequence; -, corresponding residues deleted from respective sequence; single residues depicted above the chicken uPA sequence exist between amino acids 18–19, 21–22, 59–60, 111–112, and 116–117 from the chicken tPA sequence.

Tissue-Type PA mRNA in Ovarian Follicles

Northern blot analysis using poly(A)$^+$-enriched RNA (0.5–2.0 μg) failed to detect a readily identifiable tPA transcript in either granulosa or theca tissue but did not cross-hybridize to the 2.5-kb uPA transcript (data not shown). In view of the apparent low levels of tPA mRNA transcript expressed in ovarian tissues, RT-PCR followed by Southern blot analysis was conducted using total cellular RNA collected from selected ovarian tissues and stages of follicle development. Duplicate experiments detected the presence of an amplified product, from each tissue examined, that hybridized to the chicken tPA cDNA probe (Fig. 8). Identification of this amplified cDNA as chicken tPA is further indicated by the fact that restriction digest with *Bam*HI resulted in the formation of an appropriate 510-bp digestion product.

DISCUSSION

The size of the uPA RNA transcript in ovarian tissues is estimated to be 2.5 kb, similar to that isolated from chicken embryonic fibroblasts [34]. The identification of uPA mRNA in granulosa cells is consistent with the previous identification of uPA mRNA and immunoreactive uPA in F1 follicle granulosa tissue [22, 35]. Moreover, the differential pattern of uPA mRNA expression within the granulosa layer during follicle development is in general agreement with cell-associated PA activity in that higher levels of activity are detected in prehierarchical follicles (6–8 mm, [36]; 4–7 mm, [21]) compared to preovulatory follicles.

While the likelihood that PA activity is related to follic-

TABLE 1. Homology of nucleic acid and deduced amino acid sequences of chicken tPA compared to the rat and human tPA and chicken uPA sequences.

	% versus chicken tPA[a]				
	Nucleic	Amino acid[b]			
Sequence	acid	Overall	KR 1	KR 2	CatD
Rat tPA	76.9	75.2 (89.1)	81.5 (88.9)	61.8 (79.8)	86.4 (97.0)
Human tPA	80.2	76.7 (88.6)	84.8 (90.9)	65.2 (80.9)	89.3 (98.5)
Chicken uPA	54.9	35.1 (58.4)	NA	32.6 (58.4)	43.3 (68.7)

[a] Numbers outside parentheses represent the percentage of identical amino acid matches, whereas numbers within parentheses consider conservative amino acid substitutions.
[b] KR 1, partial sequence of kringle region 1; KR 2, kringle region 2; CatD, partial sequence of catalytic domain; NA, not applicable, as chicken uPA has only a single kringle region.

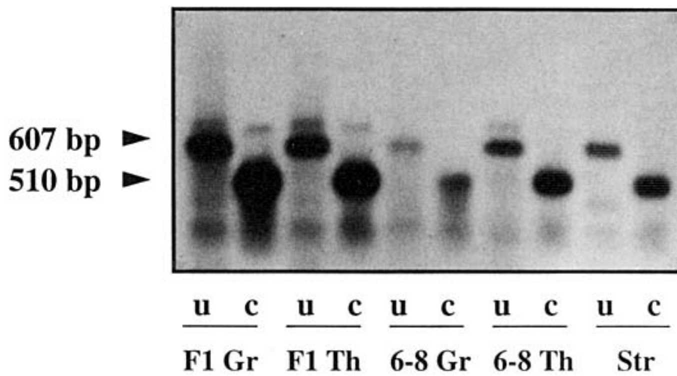

FIG. 8. Southern blot depicting tPA mRNA expression in F1 and 6–8 mm-follicle granulosa (Gr) and theca (Th) tissue, and in ovarian stromal (Str) tissue following RT-PCR amplification. The undigested (u), amplified chicken tPA product plus primers is 607 bp in length, while the *Bam*HI-digested fragment (c) is predicted to be 510 bp. Control amplifications (tPA forward and reverse primers plus polymerase without cDNA, and cDNA plus polymerase without primers) failed to hybridize with the tPA cDNA probe (data not shown). This experiment was repeated once with similar results.

ular growth and development has previously been proposed [5, 7, 36, 37], it is also recognized that the prehierarchical stage of hen follicle development is highly susceptible to undergoing atresia [38, 39]. In support of an active role for PA in mediating the process of follicle atresia is the finding that uPA mRNA levels are markedly increased in morphologically atretic compared to normal follicles (Fig. 3). While these results might seem somewhat surprising given recently published data showing that levels of other gene transcripts are significantly decreased during the progression of hen follicle atresia (e.g., *bcl*-x [29], LH receptor [32], FSH receptor [40]), they are similar to the finding of increased expression of interleukin-converting enzyme (ICE)-related proteases, ich-1 and CPP32, that occurs with atresia induced in vitro in rat antral follicles [41]. The fact that levels of the uPA transcript remain elevated during advanced stages of atresia (Fig. 3) indicates a role for this proteolytic enzyme in follicle resorption.

Several recent studies have demonstrated that hen follicle atresia is mediated via apoptosis, which is initiated predominantly within, and subsequently occurs throughout, the granulosa layer [28, 29]. If uPA activity plays a role in the progression of follicle atresia and apoptotic cell death in granulosa cells, it was hypothesized that uPA mRNA levels would increase with spontaneous programmed cell death induced by the incubation of prehierarchical-follicle granulosa cells in serum-free medium. Results of these experiments demonstrate that uPA mRNA levels are rapidly increased following the initiation of programmed cell death in apoptosis-sensitive 4- to 8-mm-follicle granulosa cells ([29]; Fig. 4A). These data suggest that an increase in uPA transcription occurs immediately upon removal from cell survival factors and provide evidence for an active role for uPA during the early stages of apoptosis prior to a significant increase in oligonucleosome formation (Fig. 5). Proposed activities might include the dissolution of extracellular matrix or the alteration of granulosa-theca interactions, as has been suggested for mammary tissues actively undergoing apoptosis [27].

By comparison, preovulatory follicles do not normally become atretic, and this is related to the fact that granulosa cells derived from such follicles are resistant to undergoing apoptosis in vitro. Thus, it was not unexpected to find that uPA mRNA levels were not altered after an 18-h incubation period. On the other hand, it has recently been demonstrated that programmed cell death in such granulosa cells can be induced within 6 h following treatment with the synthetic ceramide, C8-ceramide [33], presumably via activation of stress-activated protein kinases (mammalian cells, [42]; hen granulosa cells, J.A. Flaws and A.L. Johnson, unpublished results). The finding that C8-induced apoptotic cell death was not associated with increased uPA mRNA levels indicates that uPA expression is not mediated directly via ceramide or the stress-activated protein kinase pathway. More importantly, however, these data indicate that an immediate increase in uPA transcription appears not to be prerequisite for the progression of apoptotic cell death.

The ability of PMA treatment to increase uPA mRNA levels in preovulatory-follicle granulosa and theca tissue incubated in vitro is consistent with data reported from chick embryonic fibroblasts by Bell et al. [34]. It is of interest to note that by using nuclear run-on transcription analysis, these investigators concluded that elevated uPA mRNA levels were the result of increased gene expression and not a decreased rate of RNA degradation. Additionally, previous studies have demonstrated that PMA treatment increases PA activity in granulosa cells [18, 21] and in theca tissue [19]. It has been proposed that among the physiological factors that may serve to induce protein kinase C activation, and thus increase uPA mRNA transcription and PA activity in vivo, are prostaglandins E1 and E2 and the growth factors, transforming growth factor α, epidermal growth factor, insulin-like growth factor, and fibroblast growth factor [19, 20, 43–45]. Moreover, it is apparent from the present data that an increase in uPA mRNA alone is not sufficient to induce apoptosis, as PMA treatment of preovulatory-follicle granulosa cells, which increases levels of uPA mRNA (Fig. 2), does not directly induce programmed cell death in F1 follicle granulosa cells [33]. Accordingly, uPA may have functions unrelated to cell death within preovulatory follicles, including cellular remodeling associated with the final stages of differentiation or preparation for ovulation.

By comparison, activation of protein kinase A by treatment with 8-bromo-cAMP fails to alter levels of uPA mRNA in preovulatory-follicle granulosa cells following an 18-h incubation (Fig. 2) and does not prevent the spontaneous increase in uPA mRNA following incubation of prehierarchical-follicle granulosa cells in M199/Hepes. In contrast, activators of protein kinase A have previously been shown to significantly decrease PA activity from chick embryonic fibroblasts [46] and incubated granulosa cells [18, 23]. Therefore, the modulation of ovarian PA activity during follicle development in vivo may be mediated by FSH, LH, or vasoactive intestinal peptide, presumably via their ability to increase cellular levels of cAMP [22, 23, 47]. As this suppression of PA activity [18, 23] is not reflected by changes in levels of uPA mRNA (present study), the mode of inhibition is likely accomplished via post-transcriptional process such as altered rate of uPA translation and processing to the active enzyme or via increased activity of PA inhibitor(s) [25, 48]. It is interesting to note that after the preovulatory LH surge, uPA mRNA and immunoreactive protein as well as PA activity are all dramatically decreased, presumably in preparation for ovulation [35].

The mammalian tPA enzyme contains two kringle domains, homologous to regions of plasminogen and prothrombin [11], and a catalytic domain with three amino acids (His^{326}, Asp^{375}, Ser^{481}) that are completely conserved

within members of the serine protease family [12]. The human and rat tPAs are cleaved at an Arg-Ile-Lys processing site to produce a disulfide-linked, two-chain form of active protease [12, 49]. The chicken tPA cDNA reported herein includes a portion of the first kringle domain (27 of the 87 amino acids found in the first kringle region of the rat tPA) and the entire second kringle domain (89 amino acids) corresponding to both the rat and human tPA (Table 1); by comparison, the chicken uPA protein contains only a single kringle domain [15]. Moreover, the Arg^{130}-Ile^{131}-Lys^{132} cleavage site is completely conserved in the deduced chicken tPA sequence, indicating identical processing of this protease as compared to the rat and human tPA [12, 49]. All 13 cysteine residues, as well as a His^{177} residue corresponding to His^{326} within the rat catalytic domain, are conserved within the partial chicken tPA amino acid sequence as compared to rat tPA [12].

In the mammalian ovary, tPA is constitutively expressed at comparatively low levels; and tPA, but not uPA, mRNA and tPA activity are up-regulated by gonadotropins [7, 50]. By comparison, the hen ovary was found to contain tPA mRNA levels that were not reliably detected by Northern blot analysis. However, the tPA transcript was identifiable by Southern hybridization analysis following RT-PCR amplification, and the specificity of the Southern blot for chicken tPA is indicated by 1) the lack of nonspecific hybridization in negative controls (tPA forward and reverse primers plus polymerase without cDNA, and cDNA plus polymerase without primers) and 2) the digestion of the amplified product from chicken follicle tissues to an appropriate-length product (510 bp). With regard to the latter, neither the corresponding rat or human tPA sequence nor the chicken uPA sequences encode for a *Bam*HI restriction site. While such results cannot be interpreted with regard to translation to an active protease or even to quantitative differences among the tissues evaluated, they nevertheless establish low-level expression of the gene in the chicken ovary. In accord with these findings, zymographic studies have detected low levels of an approximately 70-kDa amiloride-insensitive enzyme, and it has been concluded that this represents tPA [25, 44].

In summary, while uPA appears to represent the major form of PA in the hen ovary, tPA mRNA (and activity) is constitutively expressed, albeit at low levels. While additional work is required to establish the relative importance of these two enzymes during follicle growth and at ovulation, it has recently been reported that expression of uPA in tPA-deficient mice is fully effective in general fibrin clearance [51]. Such results suggest that the two forms of PA exhibit considerable functional overlap and thus that their expression is not necessarily required in all tissues at equal levels. Levels of uPA mRNA during hen follicle development are related to enzymatic activity, and these data are consistent with a role for uPA in follicular maturation. Additionally, the marked increase in uPA mRNA during follicle atresia and the early stages of apoptosis in prehierarchical-follicle granulosa cells suggests a role related to the spontaneously induced progression of programmed cell death. On the other hand, apoptosis induced in otherwise resistant preovulatory-follicle granulosa cells by a ceramide analogue proceeds in the absence of elevated uPA mRNA, and an elevation of uPA mRNA induced by a pharmacologic agent (PMA) is not appropriate or sufficient to induce apoptosis. Therefore, it is concluded that the role of uPA in follicle development and cell death is dependent upon stage of maturation. Finally, a potentially broader implication related to elevated uPA mRNA levels during the early stages of programmed cell death is that specific genes may be targeted for inactivation (e.g., gonadotropin receptors, cell survival factors), while additional genes are apparently required to be activated—or at least the transcripts are stabilized to prevent degradation—to facilitate the progression of apoptotic cell death. Whether such activation/stabilization in hen granulosa cells is the result of increased gene transcription, or, alternatively, reduced mRNA degradation, remains to be determined.

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REFERENCES

1. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Neilsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 1985; 44:139–266.
2. Beers WH, Strickland S, Reich E. Ovarian plasminogen activator: relationship to ovulation and hormonal regulation. Cell 1975; 6:387–394.
3. Liu YX, Ny T, Sarak D, Loskoff D, Hsueh AJW. Identification and regulation of tissue plasminogen activator activity in rat cumulus-oocyte complexes. Endocrinology 1986; 119:1578–1587.
4. Hsueh AJW, Liu Y, Cajander XS, Peng RX, Dahl K, Kristensen P, Ny T. Gonadotropin-releasing hormone induces ovulation in hypophysectomized rats: studies on ovarian tissue type plasminogen activator activity, messenger ribonucleic acid content, and cellular localization. Endocrinology 1988; 122:1486–1495.
5. Knecht M. Plasminogen activator is associated with the extracellular matrix of ovarian granulosa cells. Mol Cell Endocrinol 1988; 56:1–9.
6. Bisak TA, Cajander SB, Peng XR, Ny T, LaPolt PS, Lu JKH, Kristensen P, Tsafriri A, Hsueh AJW. Tissue-type plasminogen activator in rat oocytes: expression during the preovulatory period, after fertilization, and during follicular atresia. Endocrinology 1989; 124:187–194.
7. Ny T, Liu YX, Ohlsson M, Jones PBC, Hsueh AJW. Regulation of tissue-type plasminogen activator activity and messenger RNA levels by gonadotropin-releasing hormone in cultured rat granulosa cells and cumulus-oocyte complexes. J Biol Chem 1987; 262:11790–11793.
8. Zhang X, Kidder GM, Zhang C, Khamsi F, Armstrong DT. Expression of plasminogen activator genes and enzymatic activities in rat preimplantation embryos. J Reprod Fertil 1994; 101:235–240.
9. Pujade-Lauraine E, Lu H, Mirshahi S, Soria J, Soria C, Bernadou A, Kruithof EKO, Lijnen HR, Burtin P. The plasminogen-activation system in ovarian tumors. Int J Cancer 1993; 55:27–31.
10. Schmitt M, Wilhelm O, Janicke F, Reuning U, Ohi H, Moniwa N, Kobayashi H, Weidle U, Graeff H. Urokinase-type plasminogen activator (uPA) and its receptor (CD87). A new target in tumor invasion and metastasis. J Obstet Gynaecol 1995; 21:151–165.
11. Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV, Collen D. Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli.* Nature 1983; 301:214–221.
12. Ny T, Leonardsson G, Hsueh AJW. Cloning and characterization of a cDNA for rat tissue-type plasminogen activator. DNA 1988; 7:671–677.
13. Nagai M, Hiramatsu R, Kaneda T, Hayasuke N, Arimura H, Nishida M, Suyama T. Molecular cloning of cDNA coding for human preprourokinase. Gene 1985; 36:183–188.
14. Henderson BR, Tansey WP, Phillips SM, Ramshaw IA, Kefford RF. Transcriptional and posttranscriptional activation of urokinase plasminogen activator gene expression in metastatic tumor cells. Cancer Res 1992; 52:2489–2496.
15. Leslie ND, Kessler CA, Bell SM, Degen JL. The chicken urokinase-type plasminogen activator gene. J Biol Chem 1990; 265:1339–1344.
16. McGuire PG, Alexander SM. Urokinase expression during the epithelial-mesenchymal transformation of the avian somite. Dev Dynam 1992; 194:193–197.
17. Valinsky JE, Reich E. Plasminogen activator in the bursa of Fabricius:

correlations with morphogenetic remodeling and cell migrations. Cell 1981; 25:471–476.
18. Tilly JL, Johnson AL. Effects of a phorbol ester, a calcium ionophore and cyclic 3′,5′-adenosine monophosphate production on hen granulosa cell plasminogen activator activity. Endocrinology 1988; 123: 1433–1441.
19. Tilly JL, Johnson AL. Control of ovarian theca cell plasminogen activator activity in the domestic hen. Endocrinology 1990; 126:2079–2087.
20. Lafrance M, Zhou L, Tsang BK. Interactions of transforming growth factor-α and -β and luteinizing hormone in the regulation of plasminogen activator activity in avian granulosa cells during follicular development. Endocrinology 1993; 133:720–727.
21. Wang L, Croze F, Morley P, Tsang BK. Granulosa-thecal cell interactions in the regulation of plasminogen activator activity during ovarian follicular development in the hen. Biol Reprod 1993; 49:924–932.
22. Jackson JA, Tischkau SA, Zhang P, Bahr JM. Plasminogen activator production by the granulosa layer is stimulated by factor(s) produced by the theca layer and inhibited by the luteinizing hormone surge in the chicken. Biol Reprod 1994; 50:812–819.
23. Johnson AL, Tilly JL. Effects of vasoactive intestinal peptide on steroid secretion and plasminogen activator activity in granulosa cells of the hen. Biol Reprod 1988; 38:296–303.
24. Tilly JL, Johnson AL. Modulation of hen granulosa cell steroidogenesis and plasminogen activator activity by transforming growth factor alpha. Growth Factors 1990; 3:247–255.
25. Politis I, Wang L, Turner JD, Tsang BK. Changes in tissue-type plasminogen activator-like and plasminogen activator inhibitor activities in granulosa and theca layers during ovarian follicle development in the domestic hen. Biol Reprod 1990; 42:747–754.
26. Brandstrom A, Westin P, Bergh A, Cajander S, Damber JE. Castration induces apoptosis in the ventral prostate but not in an androgen-sensitive prostatic adenocarcinoma in the rat. Cancer Res 1994; 54:3594–3601.
27. Lund LR, Romer J, Thomasset N, Solberg H, Pyke C, Bissell MJ, Dano K, Werb Z. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development 1996; 122:181–193.
28. Tilly JL, Kowalski KI, Johnson AL, Hsueh AJW. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 1991; 129:2799–2801.
29. Johnson AL, Bridgham JT, Witty JP, Tilly JL. Susceptibility of avian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of *bcl*-xlong gene expression. Endocrinology 1996; 137:2059–2066.
30. Tilly JL, Kowalski KI, Johnson AL. Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 1991; 44:305–314.
31. Levorse JM, Johnson AL. Regulation of steroid production in ovarian stromal tissue from 5- to 8-week-old pullets and laying hens. J Reprod Fertil 1994; 100:195–202.
32. Johnson AL, Bridgham JT, Wagner B. Characterization of a chicken luteinizing hormone receptor (cLH-R) cDNA, and expression of cLH-R mRNA in the ovary. Biol Reprod 1996; 55:304–309.
33. Witty JP, Bridgham JT, Johnson AL. Induction of apoptotic cell death in hen granulosa cells by ceramide, in vitro. Endocrinology 1996; 137: 5269–5277.
34. Bell SM, Brackenbury RW, Leslie ND, Degen JL. Plasminogen activator gene expression is induced by the *src* oncogene product and tumor promoters. J Biol Chem 1990; 265:1333–1338.
35. Tischkau SA, Jackson JA, Finnigan-Bunick C, Bahr JA. Granulosa layer: primary site of regulation of plasminogen activator messenger ribonucleic acid by luteinizing hormone in the avian ovary. Biol Reprod 1996; 55:75–79.
36. Tilly JL, Kowalski KI, Li Z, Levorse JM, Johnson AL. Plasminogen activator and DNA synthesis in avian granulosa cells during follicular development and the periovulatory period. Biol Reprod 1992; 46:195–200.
37. Karakji EG, Tsang BK. Regulation of rat granulosa cell plasminogen activator system: influence of interleukin-1 beta and ovarian follicular development. Biol Reprod 1995; 53:1302–1310.
38. Gilbert AB, Perry MM, Waddington D, Hardie MA. Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (*Gallus domesticus*). J Reprod Fertil 1983; 69:221–227.
39. Johnson AL. Some characteristics of small white ovarian follicles: implications for recruitment and atresia in the domestic hen. Ornis Scand 1992; 23:233–237.
40. You S, Bridgham JT, Foster DN, Johnson AL. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. Biol Reprod 1996; 1055–1062.
41. Flaws JA, Kugu K, Trbovich AM, DeSanti A, Tilly KI, Hirshfield AN, Tilly JL. Interleukin-1β-converting enzyme-related proteases (IRPs) and mammalian cell death: dissociation of IRP-induced oligonucleosomal endonuclease activity from morphological apoptosis in granulosa cells of the ovarian follicle. Endocrinology 1995; 136:5042–5053.
42. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 1996; 380:75–79.
43. Tilly JL, Johnson AL. Effect of several growth factors on plasminogen activator activity in granulosa and theca cells of the domestic hen. Poult Sci 1990; 69:292–299.
44. Lafrance M, Croze F, Tsang BK. Influence of growth factors on the plasminogen activator activity of avian granulosa cells from follicles at different maturational stages of preovulatory development. J Mol Endocrinol 1993; 11:291–304.
45. Li J, Li M, Lafrance M, Tsang BK. Avian granulosa cell prostaglandin secretion is regulated by transforming growth factor α and β and does not control plasminogen activator activity during follicular development. Biol Reprod 1994; 51:787–794.
46. Wilson EL, Reich E. Modulation of plasminogen activator synthesis in chick embryo fibroblasts by cyclic nucleotides and phorbol myristate acetate. Cancer Res 1979; 39:1579–1585.
47. Tilly JL Johnson AL. Presence and hormonal control of plasminogen activator factor in granulosa cells of the hen (*Gallus domesticus*). Biol Reprod 1987; 37:1156–1164.
48. Kruithof EKO. Plasminogen activator inhibitors—a review. Enzyme 1988; 40:113–121.
49. Wallen P, Pohl G, Bergsdorf N, Ranby M, Ny T, Jornwall H. Purification and structural characterization of a melanoma cell plasminogen activator. Eur J Biochem 1983; 132:681–686.
50. Liu Y-X, Cajander SB, Ny T, Kristensen P, Hsueh AJW. Gonadotropin regulation of tissue-type and urokinase-type plasminogen activators in rat granulosa and theca-interstitial cells during the periovulatory period. Mol Cell Endocrinol 1987; 54:221–229.
51. Bugge TH, Flick MJ, Danton MJS, Daugherty CC, Romer J, Dano K, Carmeliet P, Collen D, Degen JL. Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 1996; 93: 5899–5904.