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Susceptibility of Avian Ovarian Granulosa Cells to Apoptosis Is Dependent upon Stage of Follicle Development and Is Related to Endogenous Levels of *bcl*-xlong Gene Expression*

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ABSTRACT

Studies were conducted to evaluate the susceptibility of avian ovarian granulosa cells to apoptosis when incubated *in vitro* and to relate this relative susceptibility to both the stage of follicle development from which granulosa cells were collected (atresia-prone *vs.* -resistant) and to the expression of a gene previously linked to the regulation of cell viability, *bcl*-xlong. Granulosa cells from slow growing, prehierarchal (4- to 8-mm diameter; atresia-prone) follicles were found to undergo rapid and progressively extensive apoptosis after incubation in defined medium for 6–24 h ($P < 0.05$ *vs.* unincubated controls). By contrast, cells from the largest preovulatory (F1) follicle, as well as from follicles most recently recruited into the follicle hierarchy (9- to 12-mm diameter), showed significantly less low molecular wt labeling at 6 h of incubation ($P < 0.05$ *vs.* 4- to 8-mm follicles). Furthermore, the amount of low molecular wt labeling did not significantly increase in cells from either stage of follicle development at 12 or 24 h of incubation ($P > 0.05$ *vs.* 6 h incubation). This biochemical indication of ongoing apoptosis in prehierarchal follicle granulosa cells was confirmed by an increased incidence of pyknotic nuclei detected by morphological analysis. Thus, increased susceptibility to apoptosis in incubated prehierarchal follicle granulosa cells is correlated with the high rate of follicle atresia that is known to occur at this stage of development *in vivo*. Recombinant human FSH (100 mIU) and transforming growth factor-α (3.3 nM) partially suppressed apoptosis in prehierarchal follicle granulosa cells after 6 h of incubation (by 46–57%; $P < 0.05$ *vs.* control), as did the cAMP analog, 8-Br-cAMP (1 mM; by 59%; $P < 0.05$).

A single form of the *bcl*-2-related gene, *bcl*-x, was detected in hen ovarian tissues; this transcript corresponded to *bcl*-xlong, the death-suppressing form of *bcl*-x. The highest levels of *bcl*-xlong messenger RNA were found in granulosa tissue from preovulatory follicles, with significantly lower levels detected in prehierarchal follicle granulosa tissue ($P < 0.05$). Elevated expression of *bcl*-xlong in preovulatory follicles was correlated to increased resistance to the process of apoptosis, *in vitro*, and the virtual absence of follicle atresia at this stage of development, *in vivo*. We conclude that there is a direct relationship between the inherent susceptibility of avian granulosa cells to apoptosis and the high rate of follicle atresia in follicles not yet selected into the preovulatory hierarchy. Moreover, our results are consistent with the proposal that the expression of death-suppressing genes, including *bcl*-xlong, is capable of rendering cells resistant to the process of apoptosis. The findings reported herein provide the foundation for a novel model with which to further elucidate molecular mechanisms related not only to the initiation of follicle atresia, but also events associated with the process of follicle selection. (*Endocrinology* **137:** 2059–2066, 1996)

IT HAS been estimated that for every 20 hen ovarian follicles that grow to a size of 6- to 8-mm in diameter, only one will remain viable long enough to be selected into the preovulatory hierarchy; the remainder undergo atresia and become resorbed (1). In fact, it is likely that a considerably greater proportion of hen primordial follicles that begin the slow growth phase of development succumb to atresia, as estimates in mammals for the incidence of follicles undergoing atresia throughout the reproductive life span of the female is greater than 95% (2, 3).

We have previously reported that hen follicle atresia is mediated via the process of apoptosis, and that the large majority of cells undergoing this process appear to be of granulosa cell origin (4). Accordingly, genomic DNA collected from atretic follicle granulosa cells has been demonstrated to contain significant levels of internucleosomal cleavage (the hallmark of apoptosis), while granulosa cell DNA from morphologically normal follicles at all stages of development fails to exhibit a detectable presence of oligonucleosomes (4, 5). Under normal physiological and environmental conditions, once a follicle has been recruited into the preovulatory hierarchy (the final rapid growth phase preceding ovulation by 5–10 days), a negligible incidence of atresia occurs. On the other hand, natural occurrence of atresia in preovulatory follicles is observed after the onset of molt (1), while atresia in hierarchal follicles can be induced by forced-molt (6), prolonged daily administration of equine CG (7), hypophysectomy (8), or destruction of the germinal disc region (9).

Recently, there have been a number of reports implicating gonadotropins, growth factors, or other ovarian-derived fac-

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tors in the attenuation of apoptosis in incubated rat whole follicles (10–14) and isolated granulosa cells (10). Typically, prepubertal rats are primed for 48–52 h with equine CG to induce synchronous, multiple follicle growth followed by 24-h incubations with hormonal treatments. In all instances reported to date, rat follicles or isolated granulosa cells prepared as described exhibit a significant degree of apoptosis within 24 h of incubation in the absence of supportive hormonal treatment.

Research efforts in nongonadal tissues have centered on the function of protooncogene and tumor suppressor gene products as intracellular mediators of cell survival. For instance, the *bcl*-2 gene was first described as an oncogene associated with human B cell lymphoma, and its protein product prevents apoptotic cell death in a variety of cell types (for reviews, see Refs. 15 and 16). An additional member of this family, *bcl*-x, was first cloned from a chicken genomic DNA library. Two alternatively spliced forms of human *bcl*-x were subsequently identified, the longer messenger RNA (mRNA) species (*bcl*-xlong) encodes a protein that, when over-expressed in select cell types, prevents apoptosis in a manner similar to *bcl*-2, while the product of *bcl*-xshort (abbreviated by a total of 63 amino acids) counters the death repressor effects of *bcl*-2 (17). It is now recognized that the chicken *bcl*-x sequence identified corresponds to human *bcl*-xlong; a chicken *bcl*-xshort mRNA transcript or protein has not, to our knowledge, been identified.

The present experiments were conducted to compare the progression of apoptosis in granulosa cells collected from hen follicles that can be reliably classified as inherently prone (prehierarchal follicles <9-mm diameter) or resistant (preovulatory, hierarchal follicles) to undergoing atresia. Moreover, in light of mounting data that implicate products of *bcl*-2-related genes in suppressing apoptosis in a wide range of tissues (15–19), the expression of *bcl*-x mRNA was monitored relative to stage of follicle differentiation. Results of these studies demonstrate a clear relationship between stage of follicle differentiation and both the susceptibility of granulosa cells to initiate the process of apoptosis as well as to relative mRNA levels encoding the death-suppressing alternatively spliced form of *bcl*-x, *bcl*-xlong.

Materials and Methods

Animals and reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–40 weeks of age and laying regular sequences of at least five to six eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash, Purina Mills, St. Louis, MO) and water and were exposed to a photoperiod of 15-h light, 9-h darkness, with lights on at 2400 h. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. 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 PCR amplification and nucleic acid 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.

Granulosa cell incubations

Granulosa cells were collected from the largest preovulatory (F1) follicle, the follicle most recently selected into the preovulatory hierarchy (9- to 12-mm diameter), and 20–25 prehierarchal (4- to 8-mm diameter) follicles, as previously described (20). Aliquots of cells were immediately frozen at −70 C (T_0) or incubated in 12 × 75 mm polypropylene culture tubes in Medium 199/HEPES without serum (20) for 6, 12, or 24 h. In a second set of experiments, granulosa cells from prehierarchal (4- to 8-mm) follicles were immediately frozen (T_0) or incubated in the absence or presence of recombinant human FSH (rhFSH; 10 or 100 mIU; from the National Hormone and Pituitary Program), 8-Br-cAMP (1 mM), transforming growth factor α (TGFα; 0.3 or 3.3 nM; Bachem, Torrance, CA), or a well documented pharmacologic inhibitor of oligonucleosome formation, $ZnSO_4$ (1 mM; 21) for 6 h. After incubations, cells were pelleted by gentle centrifugation (200 × *g*, 5 min, room temperature) and then frozen at −70 C until extraction of genomic DNA. The collection of granulosa cells from nonatretic follicles in each instance was confirmed in retrospect by the absence of detectable oligonucleosomes from genomic DNA isolated from T_0 cell preparations.

Preparation of genomic DNA and evaluation of oligonucleosome formation

Genomic DNA was prepared as previously reported (22). After isolation and quantification of DNA, samples (1 μg/sample) were 3′-end-labeled with [α^{32}P]dideoxy-ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL) using the terminal transferase reaction, and the degree of low molecular wt (LMW; <20 kb) DNA labeled was analyzed by autoradiography, as described (4, 22).

Morphological evaluation of apoptosis

To evaluate morphological characteristics of apoptosis, granulosa cells from 4- to 8-mm follicles were collected, then immediately immersed in Dietrick's (4% formalin, 28% ethanol, 0.34 N glacial acetic acid) fixative (T_0), or incubated for 6 h in defined medium in the absence or presence of 8-Br-cAMP (1 mM). After incubation, cells were pelleted then fixed overnight in Dietrick's fixative. In addition, whole 4- to 8-mm follicles were immediately fixed in Dietrick's or were incubated for 6 h in defined medium and then fixed in Dietrick's. All tissues were subsequently embedded in paraffin, sectioned at 5 μm, then stained with Weigert's iron hematoxylin and counterstained with a picric acid/methyl blue solution. Quantification of apoptotic granulosa cells was accomplished in a blind study by determining the percent pyknotic cells in 50–100 cells from three to five random areas in each of two different incubations of granulosa cells or whole follicles.

RNA isolation and Northern blot analysis for bcl-x mRNA

Total cellular RNA was collected from granulosa and theca tissue from preovulatory (F1, F2, F3) follicles, 9- to 12-mm diameter follicles, and prehierarchal (6- to 8-mm and 3- to 5-mm) follicles. In addition, morphologically normal and atretic follicles (4- to 6-mm diameter) were collected and processed without separating granulosa and theca layers. Atretic follicles were identified based on the presence of follicle hemorrhagia, collapsed morphology, and opaque appearance. Total cellular RNA from tissue at each stage of development was isolated using Trizol Reagent (GIBCO-BRL, Gaithersburg, MD), and quantified by measuring the optical density of each sample at 260 *vs.* 280 nm. Fifteen micrograms of total cellular RNA were electrophoresed through 1% agarose gels in the presence of formaldehyde, then transferred to nitrocellulose Nitro ME membranes (MSI, Westboro, MA) by overnight capillary action.

A 485-bp chicken *bcl*-x complementary DNA (cDNA) was amplified from reverse transcribed total cellular RNA collected from F1 granulosa tissue. PCR was conducted on the resulting cDNA using primers based upon the chicken *bcl*-x sequence reported by Boise *et al.* (17) (forward primer 1: bp 35–57 from the *bcl*-x coding sequence, TTGTTTCCTACAAGCTCTCGCAG; reverse primer 1: bp 498–519 from the coding sequence, ATGGTCGGTCAAGTACGTGGTC) (Fig. 1). Amplification conditions consisted of denaturation for 1 min at 92 C, annealing for 1 min at 55 C, and elongation for 1 min at 72 C, for 35 cycles. After

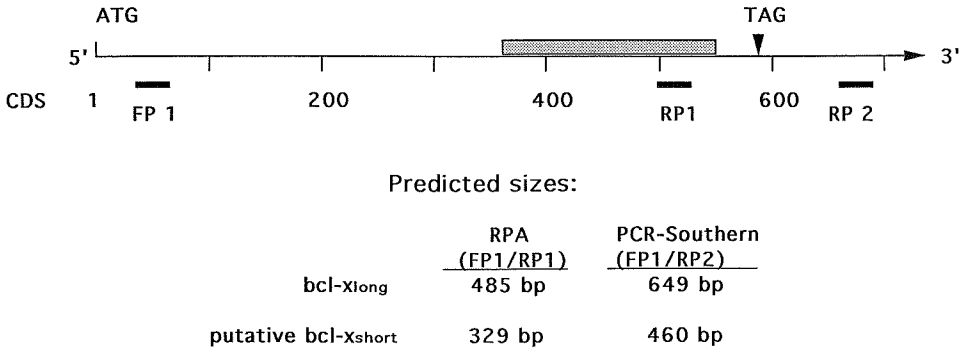

FIG. 1. Diagramatic representation of the chicken *bcl*-x cDNA (17) showing location of oligonucleotide primers used for the ribonuclease protection assay (RPA) and PCR amplification-Southern blot analysis (PCR-Southern). Predicted sizes for *bcl*-xlong and putative *bcl*-xshort fragments for each assay are described. ATG, Start codon; TAG, stop codon; CDS, Base pairs from start of coding sequence; *stippled bar* indicates sequence comparable to the alternatively spliced region from the human *bcl*-x cDNA; FP1, forward primer; RP1 and RP2, reverse primers.

amplification, the reaction mixture was separated through a 1% agarose gel and visualized by ethidium bromide staining. A product corresponding to the predicted size was isolated and subcloned into pCRII vector (Invitrogen, San Diego, CA). The amplified and cloned chicken *bcl*-x sequence was subsequently verified by nucleic acid sequence analysis using the dideoxy chain termination method (Sequenase 2.0, United States Biochemical, Cleveland, OH).

Antisense RNA probe was synthesized by *in vitro* transcription from the linearized (*Xba* I) *bcl*-x cDNA template using SP6 RNA polymerase, [α^{32}P]cytidine triphosphate (CTP) (3000 Ci/mmol; Amersham), and the Gemini II Riboprobe Core System (Promega, Madison, WI). It is noted that the *bcl*-x probe used does not distinguish between expression of *bcl*-xlong *vs.* a putative *bcl*-xshort transcript by Northern analysis. Hybridizations were conducted overnight using the cRNA probe (specific activity, 4.1×10^8 to 6.9×10^9 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 1–4 days.

Ribonuclease protection assay

A ribonuclease protection assay (RPA II kit, Ambion, Inc., Austin, TX) was used to evaluate the potential expression of two alternatively spliced *bcl*-x transcripts (*bcl*-xlong and *bcl*-xshort) in hen ovarian tissues. The 613-bp *bcl*-x antisense RNA probe (from cDNA template described above; Fig. 1) was prepared as described for Northern analysis except that the specific activity of the [α^{32}P]CTP (NEN-Dupont, Boston, MA) was 800 Ci/mmol. Briefly, 15 μg total cellular RNA were incubated with 0.6×10^6 cpm of ^{32}P-labeled *bcl*-x cRNA probe overnight at 37 C. The ribonuclease digestion was accomplished with a 1:100 dilution of ribonuclease T1 at 37 C for 30 min. Protected fragments were precipitated, resuspended in gel loading buffer, then run on a 6% acrylamide-urea gel. The predicted sizes for the *bcl*-xlong and *bcl*-xshort protected fragments are 485 bp and 329 bp, respectively; actual size was estimated from a fully characterized double-stranded sequencing reaction electrophoresed simultaneously with the protected fragments.

RT-PCR analysis of bcl-x mRNA

As a second and more sensitive method of evaluating chicken *bcl*-x mRNA for the potential expression of *bcl*-xshort, RT-PCR was conducted on total cellular RNA from preovulatory and prehierarchal follicle granulosa and theca tissue, adult brain and kidney tissue, and thymic tissue from day 19 embryos. The forward primer was the same as that described above (forward primer 1), while the reverse primer was selected to provide a product that spans the putative alternative splice site (reverse primer 2: from the 3′-noncoding sequence of chicken *bcl*-x, CCAATTTATTGCCCTGCAAGGTG) (Fig. 1). The locations of these primers were chosen to amplify a near full-length *bcl*-x product to consider the possibility that the putative splicing site in the chicken *bcl*-x transcript might not occur in the identical location as described for human *bcl*-x. Amplification conditions were as described above, and the predicted sizes for *bcl*-xlong and *bcl*-xshort products are 649 bp and 460 bp, respectively. After amplification, the DNA was separated on a 1% agarose gel, denatured and neutralized, then transferred to a Zetabind membrane (CUNO, Inc., Meridan, CT) overnight by capillary action. Membranes were prehybridized at 42 C for 18 h in a solution consisting of 5× sodium chloride-sodium citrate, 10× Denhardt's, 5% dextran sulfate, 50% formamide, and 0.5 mg/ml denatured Herring sperm. A radiolabeled cDNA probe (cDNA template was the product of forward primer 1 and reverse primer 1; Fig. 1) was generated using the Megaprime DNA labeling system (Amersham) and [α-^{32}P]dCTP (3000 Ci/mmol; Amersham), and membranes were hybridized with probe (10^6 cpm/ml hybridization solution containing 0.1 mg/ml denatured Herring sperm) overnight at 42 C. Membranes were stringently washed and exposed to autoradiographic film overnight at −70 C.

Data analysis

Northern blot analyses were repeated at least three times. The relative extent of hybridization was evaluated by densitometry (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ), and 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 house-keeping gene, is not expressed at consistent levels during hen follicle development (our unpublished observations). 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. mRNA levels (fold-increase *vs.* the appropriate control) are presented as the mean ± SEM of scanning data from replicate experiments. Data (presented as mean ± SEM) were analyzed using a paired *t* test, or by one-way ANOVA. Significant interactions ($P < 0.05$) were partitioned using the Newman-Keuls multiple range test.

Results

Susceptibility of granulosa cells to apoptosis in vitro: influence of follicular maturation

There was no difference in the extent of ^{32}P-3′-end-labeling of LMW DNA at T_0 among prehierarchal, 9- to 12-mm, and F1 follicle granulosa cells (overall mean counts per min, 454 ± 79; $P > 0.05$). By comparison, there was an increase in LMW DNA labeling in granulosa cells at all three stages of development after a 6-h incubation in defined media (Fig. 2), with the largest increase observed in granulosa from prehierarchal follicles (20.9 ± 4.3-fold increase *vs.* T_0) when compared to either 9- to 12-mm or F1 follicle granulosa cells (7.1 ± 1.5-fold or 5.1 ± 1.0-fold increase, respectively; $P < 0.05$ *vs.* prehierarchal follicles). While the degree of LMW labeling did not significantly increase in 9- to 12-mm or F1 follicle granulosa cells after 12 and 24 h of incubation (14.9 ± 2.4 and 5.5 ± 1.0-fold at T_{12} h *vs.* T_0, respectively; 12.8 ± 4.5 and 7.9 ± 3.4-fold at T_{24} h *vs.* T_0, respectively), there was a dramatic increase in LMW labeling after 12 and 24 h in prehierarchal

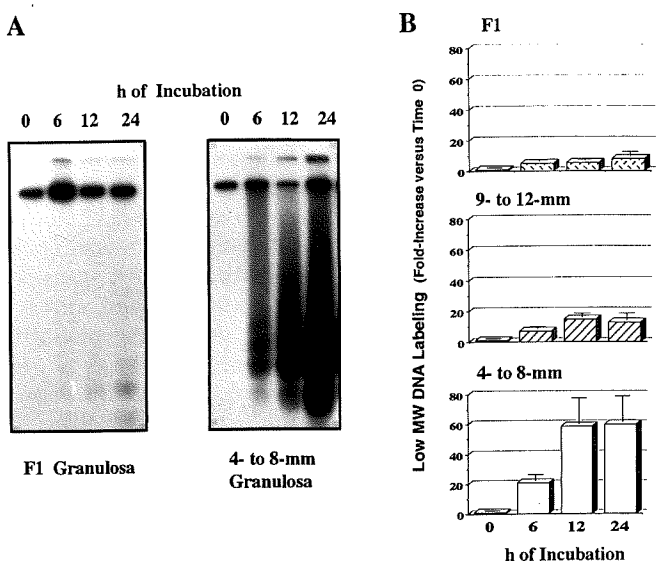

FIG. 2. A, Representative autoradiographs of oligonucleosome formation in granulosa cells from prehierarchal (4- to 8-mm diameter) follicles or the largest preovulatory (F1) follicle. Cells were isolated from morphologically normal follicles and immediately frozen at −70 C (0 h) or were incubated for 6, 12, or 24 h in Medium 199/HEPES. Genomic DNA was extracted, radiolabeled with [^{32}P]dideoxy-ATP, resolved by agarose gel electrophoresis, and then analyzed by autoradiography. B, Fold-increase in labeled LMW DNA compared to unincubated (0 h) cells determined by β-counting for LMW (<20 kb) DNA labeling (mean ± SEM, n = 3–5 replicate experiments). Cells are from prehierarchal follicles, follicles recently selected into the preovulatory hierarchy (9- to 12-mm), or the F1 follicle.

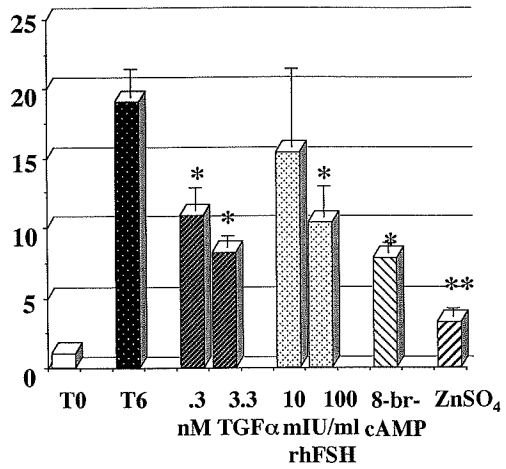

FIG. 3. Suppression of oligonucleosome formation in granulosa cells from 4- to 8-mm follicles by TGFα, rhFSH, 8-Br-cAMP (1 mM), and $ZnSO_4$ (1 mM). Granulosa cells were immediately frozen at −70 C (T_0) or incubated for 6 h in Medium 199/HEPES in the absence or presence of the above treatments, after which genomic DNA was prepared and analyzed as described in the legend to Fig. 2. Results are expressed as the mean fold-increase in LMW labeling compared to T_0 cells ± SEM (n = 4–8 replicate experiments). *, $P < 0.05$ *vs.* T6 (control); **, $P < 0.01$ *vs.* T6.

follicle granulosa cells (58.7 ± 17.7- and 60.2 ± 17.5-fold increase *vs.* T_0, respectively).

Regulation of apoptosis by physiological and pharmacological factors

In a second set of experiments, the ability of several physiological and pharmacological agents to attenuate oligonucleosome formation was evaluated. Compared to the degree of LMW labeling found in prehierarchal follicle granulosa cells after a 6-h incubation in defined medium (19.0 ± 2.1-fold increase *vs.* T_0), 10 and 100 mIU rhFSH reduced the amount of LMW labeling to 15.4 ± 5.7-fold and 10.3 ± 2.2-fold, respectively (Fig. 3). This suppressive effect was mimicked by the cAMP analog, 8-Br-cAMP (7.8 ± 0.7-fold increase). In addition, 0.3 and 3.3 nM TGFα reduced the degree of LMW labeling to 10.8 ± 1.6-fold and 8.2 ± 0.8-fold, respectively. Finally, treatment with the pharmacological inhibitor of oligonucleosome formation, $ZnSO_4$, resulted in the lowest extent of LMW labeling (3.2 ± 0.6-fold).

Morphological analysis of apoptosis induced in vitro

To confirm the biochemical evidence for the occurrence of apoptosis (*e.g.* oligonucleosome formation), the next series of experiments was designed to examine the morphological integrity of granulosa cells after a 6-h culture. To facilitate the morphological analysis, whole 4- to 8-mm follicles were used in addition to isolated granulosa cells, because intact follicles (as opposed to isolated granulosa cells) are more amenable to histological manipulations. Incubation of whole, 4- to 8-mm prehierarchal follicles for 6 h caused a dramatic increase in the incidence of pyknotic nuclei within predominantly the granulosa layer compared to unincubated controls (Fig. 4). While occasional, scattered apoptotic cells were also visable within the theca interna and externa layers, there was no apparent increase in the number of apoptotic theca cells in whole follicles after the 6-h incubation *vs.* before incubation (Fig. 4). Isolated granulosa cells incubated for 6 h in serum-free medium exhibited a higher incidence of pyknotic nuclei (32.4 ± 8.4% pyknotic nuclei; mean ± SD, n = 5 random cell counts of 50–100 cells in different cell preparations from each of two animals) compared to unincubated controls (1.4 ± 1.8%), and the incidence of pyknotic nuclei was attenuated (to 4.5 ± 3.0%) by incubation with 1 mM 8-Br-cAMP.

bcl-x mRNA expression during follicle maturation and atresia

In the final series of experiments, we sought to determine whether expression of the *bcl*-x gene changed during follicle maturation and atresia and whether changes in *bcl*-x mRNA levels were related to the susceptibility of granulosa cells to undergo apoptosis *in vitro*. Due to the fact that the difference between the long and short alternatively spliced transcripts of the human *bcl*-x is 189 bp, and thus not distinguishable by Northern blot analysis, a ribonuclease protection assay was used to determine whether chicken ovarian tissue expresses a short (putative death-inducing) form of *bcl*-x. Results of these assays provided evidence for only a single form of chicken *bcl*-x, which is comparable to human *bcl*-xlong (Fig. 5). Moreover, this result was independently confirmed from

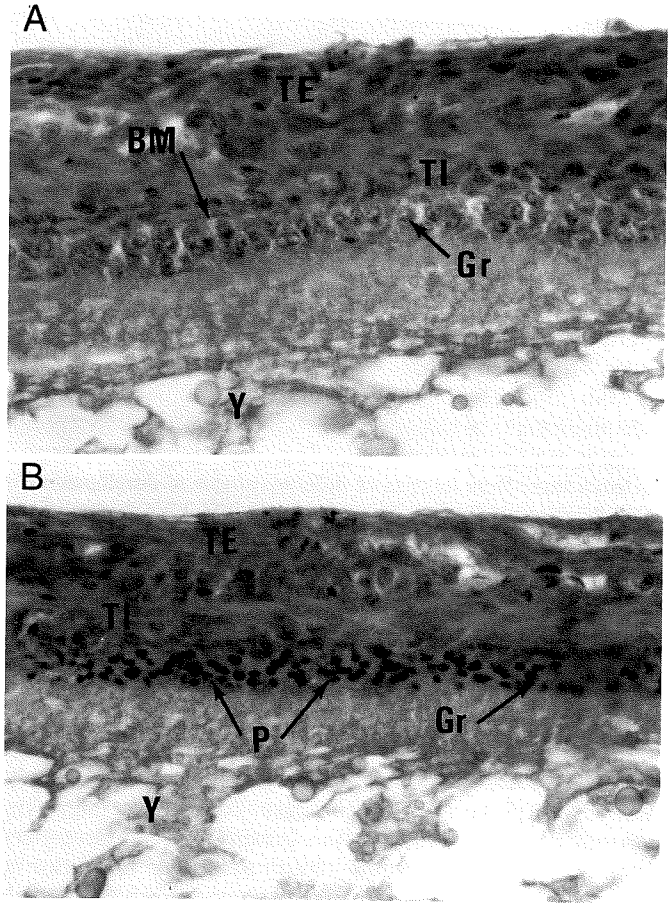

FIG. 4. Representative morphological evaluation of 4- to 8-mm follicles that were collected and immediately immersed in Deitrick's fixative (panel A), or follicles that were incubated for 6 h in Medium 199/HEPES then immersed in Dietrick's fixative (panel B). After overnight fixation, follicles were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and picric acid methyl blue stain. The incidence of pyknotic nuclei (P) within the granulosa layer (Gr) was calculated to be 1.3 ± 0.5% and 57.6 ± 3.0% in unincubated and incubated follicles, respectively. Y, Yolk; BM, basement membrane; TI, theca interna; TE, theca externa. Magnification, ×400.

a separate set of experiments using PCR amplification; amplified PCR products subsequently analyzed by Southern blot hybridization analysis revealed a predominant single 649-bp product corresponding to *bcl*-xlong (Fig. 6).

Northern blot analysis determined that levels of the 2.7-kb *bcl*-x mRNA transcript were significantly higher (by 5.0- to 9.0-fold) in granulosa cells from hierarchal follicles (including 9- to 12-mm follicles) compared to prehierarchal follicles ($P < 0.01$; Fig. 7). By comparison, there were no significant changes in *bcl*-x mRNA levels in theca tissue from follicles at any stage of development (Fig. 8). Expressed on the basis of total cellular RNA, levels of *bcl*-x mRNA were calculated to be 3.1 ± 0.5-fold higher in F1 follicle granulosa *vs.* theca tissue. Finally, levels of *bcl*-x mRNA are determined to be 3.1 ± 0.8-fold higher (n = 8 replicate observations) in whole, morphologically normal prehierarchal follicles as compared to levels of *bcl*-x mRNA in atretic follicles of the same developmental stage ($P < 0.05$; n = 5 replicate experiments).

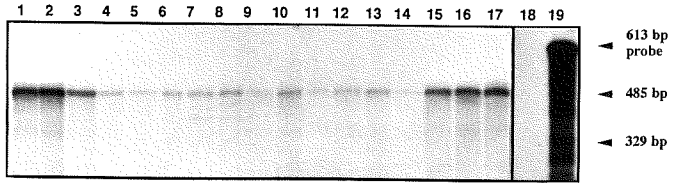

FIG. 5. Ribonuclease protection assay for *bcl*-x indicates the expression of a protected fragment representing *bcl*-xlong (485 bp), but not *bcl*-xshort (predicted 329 bp), mRNA in the hen ovary (see text and Fig. 1 for details). Lane 1, F1 granulosa total cellular RNA; lane 2, F2 granulosa; lane 3, 9- to 12-mm follicle granulosa; lane 4, 6- to 8-mm follicle granulosa; lane 5, 3- to 5-mm follicle granulosa; lane 6, F1 theca; lane 7, F2 theca; lane 8, 9- to 12-mm follicle theca; lane 9, 6- to 8-mm follicle theca; lane 10, 3- to 5-mm follicle theca; lane 11, postovulatory follicle; lanes 12–14, atretic follicles; lanes 15–17, morphologically normal, 4- to 6-mm follicles; lane 18, negative control (yeast transfer RNA); lane 19, *bcl*-x probe. This experiment was repeated once with similar results.

Discussion

The most significant result from the present studies is the identification of granulosa cells expressing two fundamentally different sensitivities to apoptosis induced by tropic hormone deprivation: granulosa cells from a population of follicles that exhibit a high rate of atresia (prehierarchal follicles < 9-mm diameter) are highly susceptible to apoptotic cell death, while granulosa cells from follicles that under normal physiological conditions do not undergo atresia (preovulatory follicles) are comparatively resistant to apoptosis. Moreover, granulosa cell resistance to apoptosis is associated with significantly higher levels of mRNA encoded by the death-suppressing gene, *bcl*-xlong, when compared to apoptosis-sensitive cells.

It is well documented that zinc ion effectively blocks the internucleosomal DNA fragmentation associated with apoptosis in mammalian (23) and avian (21) cell systems. On the other hand, there is considerable evidence that the process of apoptotic cell death, characterized by chromatin condensation, reduced cell volume, and formation of apoptotic cell bodies, can proceed in the absence of LMW oligonucleosome formation (24, 25). In fact, recent studies indicate that the formation of LMW oligonucleosomes occurs only subsequent to the cleavage of genomic DNA into at least two different pools of high molecular wt fragments [50- and 300-kb fragments (26)]. In the present study, $ZnSO_4$ was used solely as a control for nonapoptotic (*e.g.* necrotic) DNA degradation. If it is conservatively assumed that $ZnSO_4$ treatment results in the complete inhibiton of LMW oligonucleosome formation in prehierarchal follicle granulosa cells, our results indicate that a minor fraction (<15%) of LMW DNA labeling could be attributed to nonspecific DNA degradation after incubation in defined medium or to mechanical shearing during the extraction procedure.

The progressive increase in LMW DNA labeling in granulosa cells from prehierarchal follicles over the 24-h incubation period is in dramatic contrast to the relatively minor extent of DNA cleavage detected in cells from hierarchal follicles after a comparable period of incubation (<15% of the maximal oligonucleosome formation found in prehierarchal follicle granulosa cells). Also of interest is the finding that subsequent to 6 h of incubation there is no additional increase

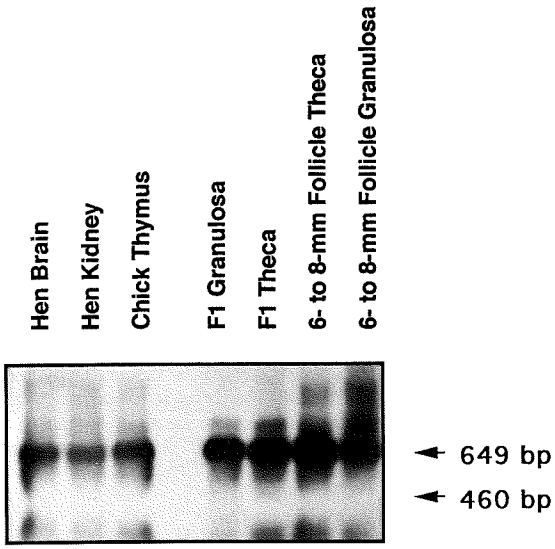

FIG. 6. Southern blot of *bcl*-x PCR products generated from forward primer 1 and reverse primer 2, which span the putative alternatively spliced region (see Fig. 1). The predicted size of *bcl*-xlong is 649 bp, and putative *bcl*-xshort is 460 bp. F1, Largest preovulatory follicle; 6- to 8-mm, prehierarchal follicles. This experiment was repeated once with similar results.

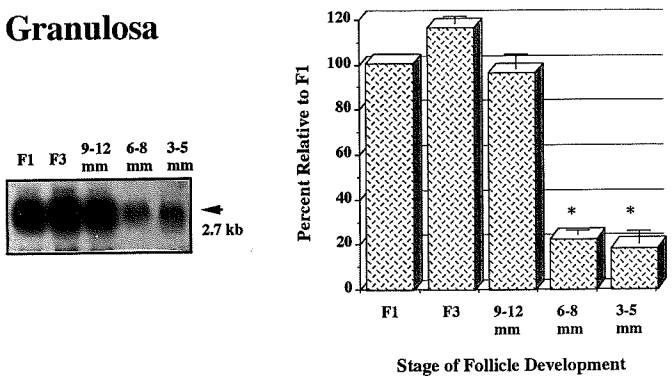

FIG. 7. A, Representative Northern blot of *bcl*-xlong mRNA levels in granulosa cells from follicles at different stages of development. B, Summary of scanning data (mean densitometric units expressed as a percent of F1 ± SEM; data from three to six replicate blots). Note that changes in hybridization intensity during follicle development are consistent with those in Fig. 5. *, $P < 0.05$ *vs.* hierarchal follicles.

in LMW labeling in F1 or 9- to 12-mm follicle granulosa cells. Previous studies of nongonadal tissues have indicated that cells actively undergoing mitosis are more susceptible to apoptosis than nonmitotic cells (27, 28). Accordingly, granulosa cells collected from the germinal disc region of hen preovulatory follicles have a higher basal rate of [^{3}H]thymidine incorporation than cells from the outer layer (29). Based upon the present data, it is suggested that in hierarchal follicles there is a small subpopulation of cells that rapidly undergoes apoptotic cell death after incubation in defined medium. Thus, it is possible that the small population of apoptosis-sensitive cells within F1 and 9- to 12-mm follicles may be derived from the mitotically active germinal disc region.

Previous studies with isolated granulosa cells or whole

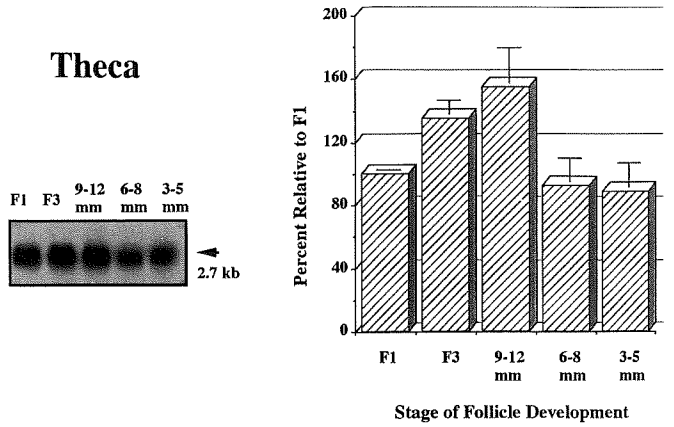

FIG. 8. A, Representative Northern blot of *bcl*-xlong mRNA levels in the theca layer from follicles at different stages of development. B, Summary of scanning data (mean densitometric units expressed as a percent of F1 ± SEM; data from three to six replicate blots).

follicles from gonadotropin-primed, prepubertal rats have demonstrated that gonadotropins (FSH and LH/hCG) and growth factors (*e.g.* TGFα, insulin-like growth factor I, fibroblast growth factor) attenuate, but do not entirely prevent, the progression of apoptotic cell death in defined medium, *in vitro* (10–14). Effects of these endocrine/paracrine/autocrine factors are mediated, at least in part, via the adenylyl cyclase/cAMP and tyrosine kinase intracellular signaling systems. Similar results in hen granulosa cells have been obtained where reduction of oligonucleosome formation to an extent of 40% to 59% of tropic hormone-deprived controls was observed after treatment with vasoactive intestinal peptide (13), 8-Br-cAMP, and the highest doses of rhFSH (100 mIU) and TGFα (3.3 nM). The lack of a complete suppression of internucleosomal cleavage in both chicken granulosa cells and rat ovarian follicles by any of the physiological factors thus far investigated perhaps indicates that multiple inhibitory mechanisms are required to function in parallel in order to maintain follicle cell viability. Consistent with oligonucleosome formation, morphological apoptosis, evidenced by a dramatic increase in the incidence of pyknotic granulosa cells, is observed after 6 h incubation of whole follicles or isolated granulosa cells in defined medium. By comparison, while 8-Br-cAMP almost entirely prevents the formation of pyknotic nuclei in granulosa cells (incubated for 6 h), it fails to prevent a significant proportion of oligonucleosome formation. A similar dissociation of biochemical *vs.* morphologic characteristics of apoptosis has been noted in incubated (for 24 h) rat follicles (13).

Results from ribonuclease protection assays plus RT-PCR and Southern blot analysis indicate that *bcl*-xlong is the primary (if not only) *bcl*-x transcript expressed in hen follicles, and this is consistent with findings from the rat ovary (19). Moreover, in contrast to the human, where the expression of *bcl*-xshort is associated with several nonovarian tissues that typically exhibit a high rate of apoptosis [*e.g.* immature thymocytes undergoing clonal deletion (17)], there is currently no evidence for an alternatively spliced *bcl*-x transcript expressed in any of the chicken tissues examined. Such data are

comparable to the reports that the long form of *bcl*-x is the predominant form of the message expressed in most mouse tissues examined (30).

Previous reports have documented the ability of Bcl-xlong protein to block apoptotic cell death in mammalian lymphocytes (17) and in a lymphoma-derived cell line (18). Therefore, the finding of elevated levels of *bcl*-xlong mRNA in granulosa cells from hen preovulatory follicles (resistant to atresia), compared to prehierarchal follicles (highly susceptible to undergoing atresia) is consistent with a proposed role for this gene product in facilitating and/or promoting granulosa cell viability. By comparison, there is no apparent relationship between levels of *bcl*-xlong mRNA and stage of follicle development in the theca layer. Previous data have suggested that apoptotic cell death during hen follicle atresia occurs primarily in the granulosa layer (4). This is supported by the dramatically increased incidence of pyknotic nuclei in granulosa cells of incubated *vs.* unincubated follicles. It is possible that constitutive expression of death-suppressing genes, including *bcl*-xlong, within the theca layer may be sufficient to render these cells comparatively more resistant to apoptosis throughout follicle development.

Considering the proposed cell-survival function for *bcl*-xlong, it is perhaps not unexpected that levels of *bcl*-xlong mRNA were found to be markedly higher in whole morphologically normal *vs.* atretic follicles. A similar reduction in mRNA levels has been reported for the LH- and FSH-receptor and cytochrome P450 aromatase enzyme mRNA transcripts, but not β-actin transcript, in porcine atretic follicles (31). As it is not possible to estimate the length of time since hen follicles had become atretic at the time of collection, it is possible that the decrease in *bcl*-x mRNA levels are due to general degradation of cellular nucleic acids. Arguing against this interpretation, however, was the finding of intact 28S and 18S ribosomal RNA bands (visualized by ethidium bromide staining) as well as a succinct band of hybridization (*i.e.* lack of smearing) for the *bcl*-x transcript in RNA from atretic follicles. Thus, it is suggested that a selective decrease in *bcl*-xlong expression is associated with, and may immediately precede, the onset of follicle atresia.

In summary, evidence has been presented to demonstrate a clear relationship between the inherent susceptibility of granulosa cells to undergo apoptosis and follicle atresia. To our knowledge this is the first identification of a stage-dependent susceptibility/resistance to apoptotic cell death within ovarian follicles of any vertebrate species. The findings reported herein provide the foundation for a novel model with which to further elucidate molecular mechanisms related not only to the initiation of follicle atresia, but also events associated with the process of follicle selection. Studies to more specifically address both of these fundamental aspects are currently ongoing.

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References

1. **Gilbert AB, Perry MM, Waddington D, Hardie MA** 1983 Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (*Gallus domesticus*). J Reprod Fertil 69:221–227
2. **Tsafriri A, Braw RH** 1984 Experimental approaches to atresia in mammals. Oxf Rev Reprod Biol 6:226–265
3. **Hirshfield AN** 1991 Development of follicles in the mammalian ovary. Int Rev Cytol 124:43–101
4. **Tilly JL, Kowalski KI, Johnson AL, Hsueh AJW** 1991 Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 129:2799–2801
5. **Johnson AL** 1992 Some characteristics of small white ovarian follicles: implications for recruitment and atresia in the domestic hen. Ornis Scand 23:233–237
6. **Breeding SW, Brake J, Garlich JD, Johnson AL** 1992 Molt induced by dietary zinc in a low-calcium diet. Poult Sci 71:168–180
7. **Johnson AL** 1986 Effects of pregnant mare serum gonadotropin (PMSG) on pituitary responsiveness to progesterone and LHRH in the hen, *Gallus domesticus*. Domest Anim Endocrinol 3:33–38
8. **Yosimura Y, Bahr JM, Okamoto T, Tamura T** 1985 Histological and histochemical observations on the atretic follicles induced by adenohypophysectomy in the hen. Jpn Poult Sci 22:134–141
9. **Yoshimura Y, Tischkau SA, Bahr JM** 1994 Destruction of the germinal disc region of an immature preovulatory follicle suppresses follicular maturation and ovulation. Biol Reprod 51:229–233
10. **Tilly JL, Billig H, Kowalski KI, Hsueh AJW** 1992 Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. Mol Endocrinol 6:1942–1950
11. **Chun S-Y, Billig H, Tilly JL, Furuta I, Tsafriri A, Hsueh AJW** 1994 Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. Endocrinology 135:1845–1853
12. **Eisenhauer KM, Chun S-Y, Billig H, Hsueh AJW** 1995 Growth hormone suppression of apoptosis in preovulatory rat follicles and partial neutralization by insulin-like growth factor binding protein. Biol Reprod 53:13–20
13. **Flaws JA, DeSanti A, Tilly KI, Javid RO, Kugu K, Johnson AL, Hirshfield AN, Tilly JL** 1995 Vasoactive intestinal peptide-mediated suppression of apoptosis in the ovary: potential mechanisms of action and evidence of a conserved antiatretogenic role through evolution. Endocrinology 136:4351–4359
14. **Tilly JL, Tilly KI** 1995 Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology 136:242–252
15. **Korsmeyer SJ** 1992 Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood 80:879–886
16. **Reed JC** 1994 Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6
17. **Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB** 1993 Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597–608
18. **Datta R, Manome Y, Taneja N, Boise LH, Weichselbaum R, Thompson CB, Slapak A, Kufe D** 1995 Overexpression of Bcl-xL by cytoxic drug exposure confers resistance to ionizing radiation-induced internucleosomal DNA fragmentation. Cell Growth Differen 6:363–370
19. **Tilly JL, Tilly KI, Kenton ML, Johnson AL** 1995 Expression of members of the bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. Endocrinology 136:232–241
20. **Tilly JL, Kowalski KI, Johnson AL** 1991 Stage of follicular devel-

opment associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 44:305–314
21. **Compton MM** 1992 A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Rev 11: 105–119
22. **Tilly JL, Hsueh AJW** 1993 Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. J Cell Physiol 154:519–526
23. **Gaido ML, Cidlowski JA** 1991 Identification, purification, and characterization of a calcium dependent endonuclease (NUC18) from apoptotic rat thymocytes: NUC18 is not histone H_2B. J Biol Chem 266:18580–18585
24. **Barbieri D, Troiano L, Grassilli E, Agnesini C, Cristofalo EA, Monti D, Capri M, Cossarizza A, Franceschi C** 1992 Inhibition of apoptosis by zinc: a reappraisal. Biochem Biophys Res Commun 187:1256–1261
25. **Flaws JA, Kugu K, Trbovich AM, DeSanti A, Tilly KI, Hirshfield AN, Tilly JL** 1995 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 136: 5042–5053
26. **Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M** 1993 Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 12:3679–3684
27. **Colombel M, Olsson CA, Ng P-Y, Buttyan R** 1992 Hormone-regulated apoptosis results from reentry of differentiated prostate cells onto a defective cell cycle. Can Res 52:4313–4319
28. **Evans DL, Dive C** 1993 Effects of cisplatin on the induction of apoptosis in proliferating hepatome cells and nonproliferating immature thymocytes. Can Res 53:2133–2139
29. **Tilly JL, Kowalski KI, Li Z, Levorse JM, Johnson AL** 1992 Plasminogen activator activity and thymidine incorporation in avian granulosa cells during follicular development and the periovulatory period. Biol Reprod 46:195–200
30. **Gonzalez-Garcia M, Perez-Ballestero R, Ding L, Duan L, Boise LH, Thompson CB, Nunez G** 1994 bcl-xL is the major *bcl*-x mRNA form expressed during murine development and its product localizes to mitochondria. Development 120:3033–3042
31. **Tilly JL, Kowalski KI, Schomberg DW, Hsueh AJW** 1992 Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 aromatase. Endocrinology 131:1670–1676